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### Research Article

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# Interaction of Human Monocytes, Macrophages, and Polymorphonuclear Leukocytes with Zymosan In Vitro

## Role of Type 3 Complement Receptors and Macrophage-derived Complement

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### Abstract

Macrophages take up zymosan in the absence of exogenous complement via receptors for iC3b (type 3 complement receptors) acting with or without lectin-like receptors for mannosyl-fucosyl-terminated glycoconjugates. We previously provided evidence that macrophages themselves secrete complement-alternative pathway components able to opsonize zymosan locally (Ezekowitz et al., *J. Exp. Med.* 1984. 159:244–260). We show here that covalently bound C3 cleavage products C3b and iC3b can be eluted from zymosan particles cultivated with 36-h adherent human monocytes in the absence of serum. The ligand binding site of type 3 complement receptors is involved in macrophage–zymosan interactions as shown by inhibition studies of zymosan binding and uptake with Fab fragments of anti-C3 antibodies and monoclonal antireceptor antibodies M01 and OKM10. In contrast, antibody IB4, which binds to a receptor epitope distinct from the binding site, does not inhibit zymosan uptake. Selective modulation of macrophage receptors onto anticomplement receptor antibody and mannose-rich yeast mannan, respectively, confirms that the complement and lectin-like receptors are distinct. Human polymorphonuclear leukocytes, which express receptors for complement, but are not known to secrete complement proteins, bind and ingest only exogenously opsonized zymosan. Unopsonized zymosan is a poor trigger of respiratory burst activity in neutrophils or 7-d adherent human macrophages, but induces cell aggregation and secretion of large amounts of superoxide anion when these cells are co-cultivated in serum-free medium and challenged with zymosan. Our studies indicate that complement and/or other products synthesized by macrophages at extravascular sites could play an important role in opsonization and lysis of pathogens able to activate the alternative pathway and mediate macrophage–neutrophil collaboration in first-line host defence.

### Introduction

Various macrophage (M $\phi$ )<sup>1</sup> populations bind and ingest zymosan in the absence of an exogenous opsonin (1). Monoclonal anti-

bodies directed against the functional site of the type 3 complement receptor (CR3) for iC3b-coated particles (2, 3) partially inhibit uptake of unopsonized zymosan. Direct sugar recognition by the lectin-like mannosyl-fucosyl receptor on macrophages (MFR[4]) has also been implicated in zymosan uptake by use of mannan as inhibitor, and additive effects of MFR and CR3 inhibitors were noted in some M $\phi$  populations. We and others (5–7) have presented evidence that M $\phi$  themselves produce complement components of the alternative and classical pathways. Biosynthetic labeling indicated that M $\phi$  synthesize and secrete active C3 under assay conditions, and C3 convertase components, factor H and I activities, were detected in M $\phi$ -conditioned medium. We therefore postulated that locally secreted complement components could opsonize targets such as zymosan for uptake via CR3. Studies by Blackwell et al. (8) have provided evidence that a similar mechanism contributes to the initial penetration of resident peritoneal mouse M $\phi$  by promastigotes of *Leishmania donovani*, which also activate the alternative pathway of complement.

In this article we provide further evidence that the ligand iC3b is deposited on zymosan under assay conditions and examine the role of CR3 in zymosan uptake by using monoclonal antireceptor antibodies. Because polymorphonuclear leukocytes (PMN) express CR3, but are not known to secrete complement proteins, we have studied their interaction with unopsonized and iC3b-opsonized zymosan. We, like others (9, 10), find that human PMN only take up zymosan after exogenous opsonization. Co-cultivation with M $\phi$ , however, enables PMN to respond to zymosan by a vigorous respiratory burst indicating that M $\phi$  complement and/or other products enable the PMN to react with zymosan particles in the absence of serum. This in vitro system indicates a novel role for the M $\phi$  in interaction with PMN and initiation of the acute inflammatory response.

### Methods

#### Mice

CBA T<sub>6</sub>T<sub>6</sub> were bred at the Sir William Dunn School of Pathology, Oxford, and both sexes were used at 20–30 g.

#### Media and reagents

Iscove's modification of Dulbecco's medium (IM) was obtained from Gibco-Biocult Ltd., Paisley, Scotland. Fetal bovine serum (FBS) from the same source was routinely heat-inactivated (56°C for 30 min) before

1. *Abbreviations used in this paper:* CR3, type 3 complement receptor; E, sheep erythrocyte; EA, sheep erythrocyte coated with rabbit IgM anti-erythrocyte antibody; FBS, fetal bovine serum; IM, Iscove's modified medium; KRPG, Krebs–Ringer phosphate buffer with glucose; Man-BSA, mannosylated bovine serum albumin; MFR, mannosyl-fucosyl receptor; M $\phi$ , macrophage; NBT, nitroblue tetrazolium; O<sub>2</sub><sup>-</sup>, superoxide ion; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; RPM, resident peritoneal macrophage.

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use. 100 µg/ml kanamycin, 50 µg/ml streptomycin, and 50 µg/ml penicillin were added to media. Phosphate-buffered saline (PBS) A and B were obtained from Oxoid Ltd., Basingstoke, England. Ficoll-Paque was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Mannan, ribonuclease B, zymosan type A, cytochrome *c*, superoxide dismutase, phorbol myristate acetate (PMA), and proteins used as molecular weight standards were all obtained from Sigma Chemical Co., St. Louis, MO.

### Antibodies

The rat anti-mouse hybridoma M1/70 (anti-MAC-1), used as a tissue culture supernatant at saturation or as an Fab fragment, was a kind gift from Dr. T. Springer, Harvard Medical School, Boston, MA. This antibody defines the CR3 on mouse Mφ, PMN, and natural killer cells (2). M01 (3) and OKM10 (11) are mouse anti-human antibodies and were gifts of Dr. R. Todd, Harvard Medical School, and Dr. G. Goldstein, Ortho Pharmaceuticals, Rahway, NJ, respectively. Both antibodies inhibit binding and uptake of iC3b ligands and were used as ascites at saturating concentrations. The monoclonal antibody, IB4, which was a kind gift of Dr. S. D. Wright, The Rockefeller University, New York, is directed against a different epitope of the human iC3b receptor (11). Rabbit anti-human C3 was prepared and converted to the Fab fragment as described (12).

### Cells

**Mouse macrophages.** Resident peritoneal macrophages (RPM) were harvested from unstimulated mice (1). The cells were washed, suspended in IM, and plated on glass, mannan-coated coverslips, or M1/70-coated coverslips at  $1 \times 10^5$  Mφ per coverslip.

**Isolation of human monocytes and PMN.** Dextran T250 3% wt/vol was added to fresh whole blood from the John Radcliffe Hospital Blood Transfusion Service, Headington, Oxford, and mononuclear cells and PMN were isolated by centrifugation on Ficoll-Paque. The mononuclear fraction was washed five times in IM at 200 *g* to remove platelets. Cells were resuspended in serum free IM and adhered for 1 h in 24-well tissue culture trays and washed to remove nonadherent cells, and IM + 10% heat-inactivated human serum pooled from 20 different donors were added. The cells were cultivated for 24 h or 7 d. Cells were cultivated on glass coverslips for phase-contrast microscopy or as described below for electron microscopy. During cultivation for 7 d cells became well spread and resembled mature Mφ morphologically. To remove serum proteins prior to assay, all cultures were washed five times in PBS and then cultivated for a further 2 h in serum-free IM, which was replaced immediately before assay. PMN were obtained by exposing the pellets from the Ficoll-Paque gradients to two treatments with hypotonic lysis buffer (0.2% wt/vol NaCl) to lyse erythrocytes. Cells were resuspended in IM or Krebs-Ringer phosphate buffer solution with glucose (KRPG) and either assayed in suspension or adhered for 15 min as described below. 99% of cells were PMN and >98% excluded trypan blue.

### Ligands

A glycoconjugate of mannan-bovine serum albumin (Man-BSA) with 33–37 mol of sugar/mol of protein was a gift from Dr. P. Stahl, Washington University, St. Louis, MO. This material was trace-labeled with  $^{125}\text{I}$  and used at  $8 \times 10^3$  cpm/ng. Zymosan was boiled for 15 min in 10 ml of PBS and was pelleted by centrifugation. Zymosan was iodinated as described (13).

### Complement

Human C3 was prepared as previously described (14). Factors H and I, respectively, were isolated as previously described (15, 16).

Sensitized erythrocytes (E) bearing C3 fragments (C3b and iC3b) were prepared as previously described (12). The IgM fraction from rabbit hemolysin was used to sensitize the erythrocytes (EA).

Zymosan coated with human C3b was prepared as described (17) and converted to zymosan-iC3b by incubation for 4 h at 37°C in PBS containing 10 µg/ml factor H and 2 µg/ml factor I. Factors H and I were removed by washing in PBS. The zymosan had 14,000 C3b or iC3b sites/particle.

### Preparation of mannan-coated and M1/70-coated coverslips

Glass coverslips were boiled in decon for 5 min and washed extensively in running tap water. Proteins were covalently coupled as described (18). Briefly, surfaces were treated with poly-L-lysine, with glutaraldehyde, then with the Fab fragment of M1/70 at a concentration of 50 µg/ml. After 90 min at 20°C the unreacted glutaraldehyde was neutralized with 0.1 M glycine (pH 7.5) containing human serum albumin at 1 mg/ml. Mannan-coated coverslips were prepared as described (19). 50 mg/ml mannan and 10 mg/ml carbodiimide hydrochloride were layered on poly-L-lysine-coated coverslips overnight at 4°C.

### Deposition of cleaved C3 on zymosan

Zymosan particles were incubated under serum-free conditions with human monocytes in the presence of inhibitors of uptake, recovered, washed free of noncovalently bound material and then stripped to remove ester-bound C3 fragments. Before zymosan challenge the monocytes ( $\sim 5 \times 10^7$ ) were cultivated for 24–36 h in medium containing 10% heat-inactivated pooled human serum, washed five times, incubated for 2 h in serum-free medium to remove adsorbed proteins and incubated further for 6 h in fresh serum-free medium. Monolayers were then incubated for 30 min at 37°C with well-suspended zymosan particles,  $\sim 100$  per cell, in the presence of anti-CR3 monoclonal antibody and mannan. The zymosan particles were carefully collected and washed successively with 2 M NaCl, water, 1% SDS, 1% Triton X-100 in 150 mM NaCl, and finally in 6 M guanidine-HCl (pH 7.0) to remove noncovalently bound material. The material was then suspended in physiologic saline, frozen, and thawed, rewashed in 0.2 M Tris-2% SDS-8 M urea, pH 8.0, and finally incubated for 3 h at 37°C in 200 µl 50 mM diethanolamine-HCl (pH 11.5)-100 mM NaCl-0.1% SDS. The last incubation conditions remove ester-bound C3 fragments from carbohydrate surfaces (14). The high-pH eluate from the zymosan was radioiodinated with  $^{125}\text{I}$  using chloramine T to facilitate detection of protein in the eluate. The radioiodinated sample was then reduced with dithiothreitol, run on SDS polyacrylamide gels, and compared with C3 fragment standards.

### Secretion of C3 and factor H

1-d monocyte cultures were prepared as above, washed, and incubated 2 h in serum-free medium. Cells were then incubated for 12 h in methionine-free medium containing 1% bovine serum albumin and [ $^{35}\text{S}$ ]methionine, 250 µCi/ml. Conditioned medium was collected, cleared by centrifugation, and processed as follows. 2.5 ml of supernatant containing  $3.5 \times 10^6$  dpm of  $^{35}\text{S}$  incorporated into nondialyzable material was mixed with 1 ml of human plasma and fractionated on a  $27 \times 1.8$ -cm diam column of DEAE-cellulose equilibrated in 25 mM potassium phosphate-5 mM EDTA-50 mM  $\epsilon$ -aminocaproate, pH 7.0.  $\sim 40\%$  of the radioactivity was retained on the column and this was eluted by applying a linear gradient (300 ml) of NaCl, from 0 to 0.35 M. This chromatographic procedure is a standard preparative step for C3 and factor H and the elution positions of C3 and H are well characterized (20). Cold carrier C3 and factor H in the column eluate were detected by Ouchterlony immunodiffusion using monospecific antisera. The proteins eluted in the expected positions. Portions of successive column fractions were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The gels were treated with Autofluor (National Diagnostics, Inc., Somerville, NJ) and the  $^{35}\text{S}$ -labeled proteins were detected by fluorography.

### Assays

**Uptake of  $^{125}\text{I}$ -zymosan.** Human 1-d cultured monocytes were preincubated with or without saturating amounts of anti-CR3 antibodies, the Fab fragment of anti-C3 globulin or 1 mg/ml yeast mannan in IM for 15 min at 37°C in 5%  $\text{CO}_2$ . After a further incubation with  $^{125}\text{I}$ -zymosan ( $\sim 100$  zymosan particles/cell), the cells were washed five times to remove free zymosan. Attached zymosan was removed by a 10-min treatment with 2.5% wt/vol trypsin solution (Gibco Biocult Ltd.) at 37°C as described (1). Cells were then solubilized in 1 M NaOH and cell-associated

radioactivity was measured in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, IL).

**Mannose-specific endocytosis.** Uptake was measured using saturating amounts of trace-labeled Man-BSA as described (21) and was inhibited by yeast mannan (1 mg/ml). Results were expressed as nanograms ligand/microgram of cell protein.

**Binding of EAiC3b by RPM on mannan- or M1/70-coated coverslips.** M $\phi$  on coverslips were placed in 0.5 ml of IM in wells. 50  $\mu$ l of EA coated with iC3b (26,500 sites/cell) was added. After 45 min at 37°C, nonattached E were removed, the M $\phi$  were fixed in 0.25% glutaraldehyde in PBS, and attached E were counted by phase-contrast microscopy.

**Uptake of zymosan by human M $\phi$  and PMN.** Human blood monocytes were cultivated for 7 d in IM + 10% heat-inactivated human serum on sterile glass chips, washed extensively, and cultivated for a further 2 h in IM, which was replaced with fresh medium prior to addition of 10  $\mu$ l of 1 mg/ml boiled unopsonized zymosan. After 10 min at 37°C, unbound zymosan particles were removed by extensive washing. Freshly isolated human peripheral blood PMN were adhered for 15 min in IM to tissue culture plastic dishes which had been preincubated with 10% heat-inactivated human serum as above and coated with 25  $\mu$ g/ml poly-L-lysine. Unopsonized or iC3b-coated zymosan particles were added for 15 min at 37°C.

### Electron microscopy

**Scanning.** Glass chips with adherent cells were fixed for 30 min in 2% glutaraldehyde in 0.1 M cacodylate buffer containing 1% sucrose. They were washed in buffer, postfixed in 1% osmium tetroxide for 45 min, dehydrated in graded alcohols, and critical point dried (Polaron Equipment Ltd., Watford, England). After sputter coating with gold (Polaron E5100), they were examined in a JEOL 100 CX electron microscope (JEOL, London, England) with ASID scanning attachment operating at 40 kV.

**Transmission.** Adherent PMN were fixed in situ in glutaraldehyde as above. The monolayer was recovered with propylene oxide, washed in propylene oxide, pelleted, dehydrated, and embedded in Araldite. Sections were cut with a diamond knife, stained with lead citrate, and examined in a JEOL 100 CX microscope operating at 80 kV.

### Respiratory burst assays—superoxide anion ( $O_2^-$ ) release

PMN and freshly isolated monocytes were assayed in suspension as described (22). Superoxide dismutase-inhibitable reduction of ferricytochrome *c* was measured spectrophotometrically after incubation of cells with unopsonized or serum-opsonized zymosan  $\sim$ 100 particles per cell, or PMA. Assays with PMN were terminated after <10 min of incubation that was found to give optimal reduction, before significant reoxidation of cytochrome *c*; monocyte assays were continued for up to 60 min. Cultivated M $\phi$  (7 d) and PMN (15 min) were also assayed with various stimuli after adherence to glass coverslips. With PMN the coverslips were preincubated for 7 d with IM + 10% heat-inactivated human serum and washed well to reduce adherence-induced release of  $O_2^-$ .

### Nitroblue tetrazolium (NBT) reduction

7-d human M $\phi$  and 15-min adherent PMN on glass coverslips were incubated in KRPG alone or together, with or without unopsonized zymosan. Pretreated glass coverslips were used for PMN alone. 200 mg of NBT was resuspended in 1 ml of KRPG and centrifuged in a microfuge for 3 min. The supernatant was filtered through a 0.22- $\mu$ m Millipore filter and 30  $\mu$ l was added to each preparation, with or without 5  $\mu$ l of 1 mg/ml zymosan. After 15 min at 37°C, the cells were washed extensively with PBS, fixed in 0.25% glutaraldehyde in PBS and viewed by phase-contrast and bright-field microscopy.

## Results

### Biochemical evidence that iC3b is deposited on zymosan and confirmation of C3 and factor H secretion by human monocytes

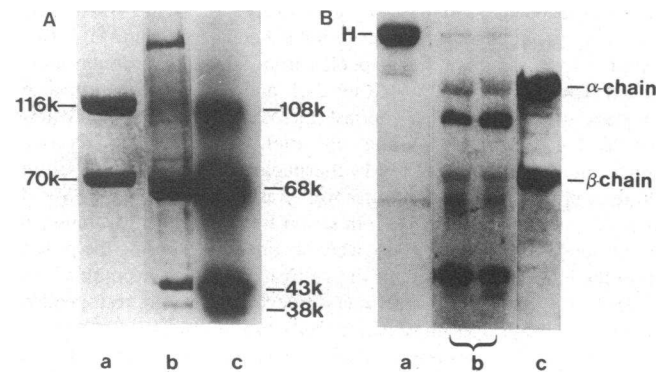
We sought direct evidence that iC3b, a known ligand of the CR3 (2), was present on zymosan particles that had been exposed to

monocytes/M $\phi$  without serum and with inhibitors present to prevent uptake. We took advantage of the covalent interaction between activated C3 and zymosan to elute bound C3 cleavage products selectively. Fig. 1 *A* shows that the zymosan eluate contained polypeptides that co-migrate with the three chains of iC3b: 43,000, 68,000, and 70,000 mol wt. In addition, it contains material (108,000 mol wt) co-running with the C3 $\beta$  $\alpha'$ -chain and a characteristic 38,000-mol wt degradation fragment of the iC3b 43,000-mol wt chain. The result is consistent with deposition of C3b on the zymosan surface, followed by degradation of over 50% of the C3b to iC3b.

In previous studies we demonstrated the presence of native C3 in human monocyte culture supernatant by biosynthetic labeling and found low levels of factor I plus cofactor activity (? factor H) by measuring iC3b formation from C3b (1). To establish that monocytes synthesize and secrete factor H under assay conditions, we partially purified  $^{35}$ S-labeled factor H and C3 from 1-d cultured monocyte-conditioned medium by ion-exchange chromatography. Analysis by SDS-PAGE in both reducing and nonreducing (not shown) conditions (Fig. 1 *B*) indicated that both proteins were detectable by fluorography, compared with standards. These were minor labeled components in that C3 contained <3% of the protein-bound  $^{35}$ S, whereas factor H contained <0.25%.

### Role of C3 in uptake of zymosan by monocytes/M $\phi$

Previous studies (1) showed that M01, a mouse anti-human CR3 monoclonal antibody, inhibited uptake of  $^{125}$ I-zymosan by human monocytes and 7-d cultured M $\phi$ . To extend these obser-



**Figure 1.** (A) SDS-PAGE of material eluted from zymosan and radioiodinated as described. Samples reduced before electrophoresis. (Tracks *a* and *b*) Coomassie blue-stained standards; (track *c*) autoradiograph. (Track *a*) C3  $\alpha$ - and  $\beta$ -chains. (Track *b*) C3b digested with factor H (15% wt/vol) and factor I (1% wt/vol) to form iC3b. The uppermost band is factor H. Track *c* shows the chains of iC3b (43,000 and 68,000–70,000-mol wt doublet which is unresolved) and C3 $\beta$  $\alpha'$ , a 108,000-mol wt cleavage product of C3b. The 38,000-mol wt degradation fragment of the 43,000-mol wt chain of iC3b is also present in the standard. K, thousands in molecular weight. (B) SDS-PAGE (reduced) of  $^{35}$ S-labeled factor H and C3 in the eluate from a DEAE-cellulose column. The elution position of the two proteins overlapped and a fluorograph of adjacent column fractions is shown in both tracks labeled *b*. Tracks *a* and *c* show Coomassie blue-stained purified factor H and C3, respectively. In addition to factor H and C3  $\alpha$ - and  $\beta$ -chains, the tracks labeled *b* also contain other prominent components (95,000, 58,000, 43,000, and 38,000 mol wt). The 95,000-mol wt band resembles on the basis of size and charge a gelatin-binding monocyte secretion product described by Vartio et al. (23); the other products have not been identified.

Table I. Effects of Anti-CR3 Antibodies and Anti-C3 Fab on Uptake of Unopsonized Zymosan by Human Monocytes

Cells	Ingestion of <sup>125</sup> I-zymosan  cpm	Percent inhibition of uptake after treatment with				
		M01	OKM10	IB4	Anti-C3 Fab	Mannan
1-d cultivated human monocytes	10,942±690	70±10	64±6	10±6	38±6	12±4

Human monocytes were cultivated for 24 h in 24-well tissue culture trays in IM + 10% heat-inactivated human serum. 2 h prior to assay the cells were washed free of serum and reincubated with IM alone. Monolayers were incubated in the presence or absence of inhibitors for 15 min prior to incubation with <sup>125</sup>I-zymosan for 10 min at 37°C in the continuous presence of inhibitor (1). Attached zymosan was removed by 15 min of trypsin treatment (1). Results show mean±standard deviation of triplicate wells from one experiment, representative of five independent experiments.

variations we studied inhibition of zymosan uptake by two other anti-CR3 antibodies (11), OKM10 and IB4, and by the Fab fragment of a rabbit anti-human C3 antibodies. OKM10, like M01, recognizes the ligand binding site of CR3, whereas IB4 binds to an epitope remote from the binding site (11). The results show (Table I) that M01 and OKM10 antibodies inhibited zymosan ingestion by adherent monocytes by 70% and 64%, respectively, whereas inhibition by IB4 was 10% of untreated controls. The anti-C3 Fab inhibited 38% of uptake. Mannan inhibition in these experiments was low in keeping with the low levels of MFR expressed by 1-d cultivated monocytes compared with cells cultivated for 7 d (1, 24). Indirect binding assays confirmed that all monoclonal antibodies bound to the cells (results not shown). These inhibition studies indicate that the uptake of zymosan by recently isolated monocytes is in large part mediated by the CR3 ligand binding site and together with the results shown in Fig. 1 are strongly suggestive that the ligand is indeed iC3b deposited on the zymosan.

#### CR3 and MFR are distinct receptors

Earlier studies showed that zymosan that had not been opsonized with fresh serum retained much of its biological activity (22) and that the MFR contributed to uptake of these particles (19). Uptake of "unopsonized" zymosan by mononuclear phagocytes can be mediated by CR3 as well as MFR depending on the expression of each receptor by particular Mφ populations (1). Recent studies show that both receptors are required for uptake of *Leishmania donovani* promastigotes (8).

To determine whether these receptors can function as distinct molecules in phagocytic recognition, we performed experiments in which each receptor was selectively depleted from the Mφ plasma membrane by adherence to ligand-coated substrata as described by Michl et al. (18). Receptor activities were measured by the ability to rosette defined EAiC3b and as specific, mannan-inhibitable uptake of <sup>125</sup>I-Man-BSA. Resident peritoneal Mφ from the mouse were selected because these cells express both MFR and CR3 and are suitable nonadherent cells for modulation experiments. RPM were cultivated on glass coverslips which had been coated with the Fab fragment of rat anti-mouse CR3 (MAC-1 antigen) or mannan. RPM adhered on M1/70 Fab surfaces selectively lost the ability to rosette EAiC3b, whereas MFR activity was similar to that of cells adhered to control substrata (Fig. 2 A and legend). The reciprocal experiment (Fig. 2 B) showed that Mφ- on mannan-coated coverslips expressed reduced MFR activity, but EAiC3b rosetting was similar to controls. There was no loss of MAC-1 antigen from the surface of Mφ on a mannan surface as determined by indirect binding

studies using antireceptor antibodies (not shown). Neither the CR3 nor MFR was modulated on Mφ cultivated on coverslips coated with the Fab fragment of 2.4G2, a rat anti-mouse Fc

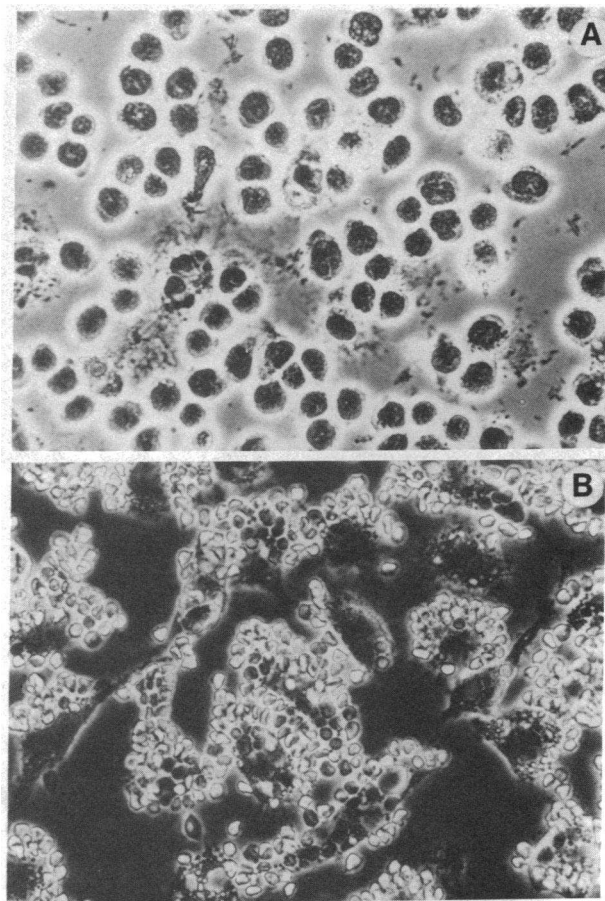


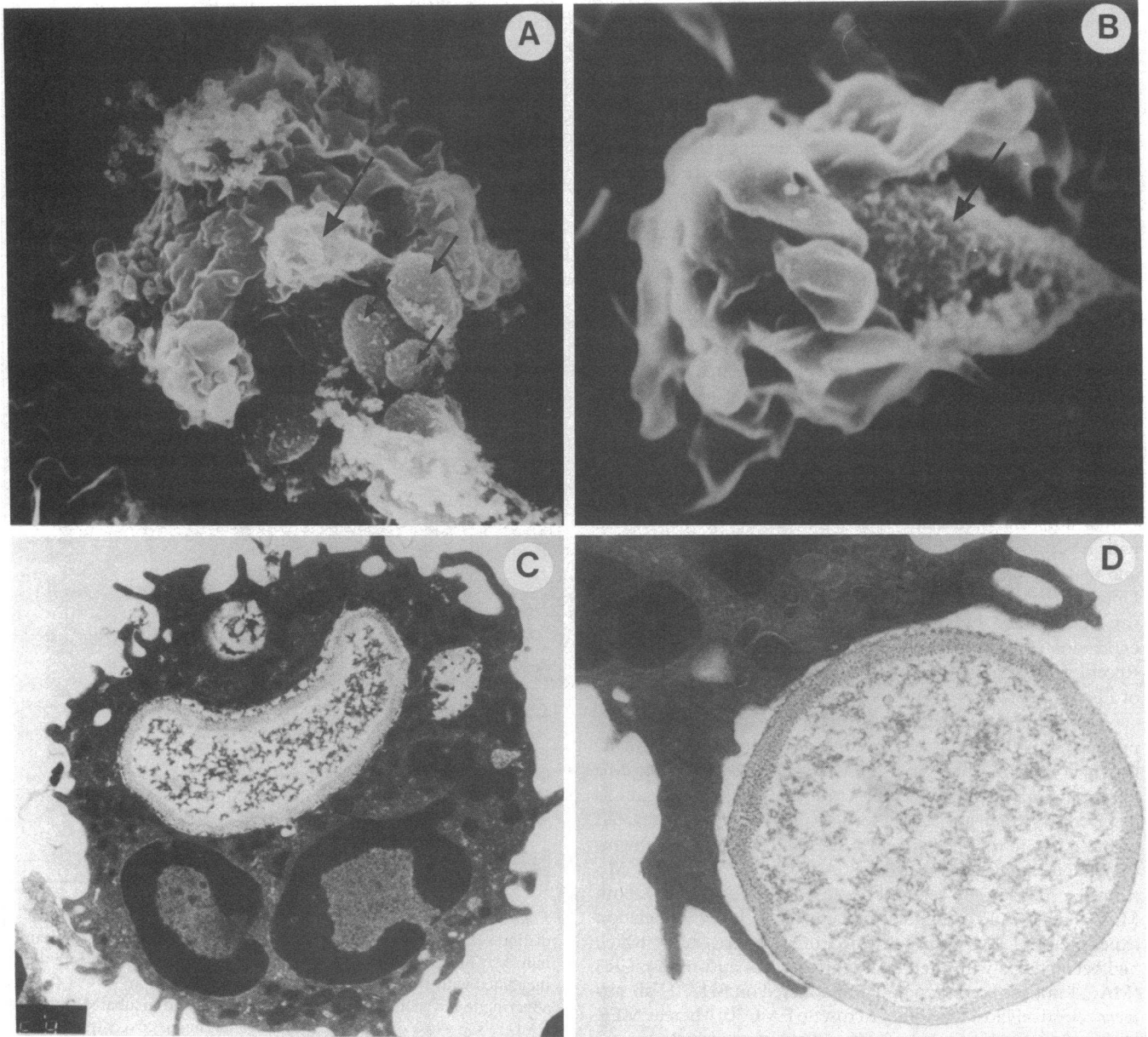
Figure 2. Mφ receptors for mannosyl-glycoconjugates and iC3b are distinct. RPM were cultivated for 4 h on coverslips coated with mannan, M1/70 Fab (anti-CR3), or on control coverslips. They were then incubated with <sup>125</sup>I-Man-BSA or EAiC3b for 30 min at 37°C. (A) Mφ adhering to coverslips coated with M1/70 Fab and incubated with EAiC3b show loss of rosetting. (B) Mφ adhering to coverslips coated with mannan and incubated with EAiC3b shows strong rosette formation. Specific uptake of <sup>125</sup>I-Man-BSA by Mφ cultivated on mannan was 0.20 ng/μg cell protein compared with 0.82 ng/μg cell protein by Mφ on M1/70 or control coverslips. Results shown are representative of six coverslips from one experiment and are representative of three independent experiments.

receptor (trypsin resistant) monoclonal antibody (25), although the Fc receptor was selectively modulated (not shown). Therefore MFR and CR3 can be selectively modulated and are distinct from each other and the trypsin-resistant Fc receptor.

*Human PMN bind and ingest opsonized, but not unopsonized zymosan*

PMN express receptors for the cleaved components of active C3, C3b (CR1) (26), and iC3b (CR3) (11) and monoclonal anti-CR3 antibodies immunoprecipitate antigens which are identical to M $\phi$  CR3 (11). These cells unlike M $\phi$  have not been shown to secrete complement proteins and can be used to test whether interaction of zymosan with CR3 is direct or dependent upon

an opsonin. We compared uptake of unopsonized and iC3b-coated zymosan by freshly isolated PMN and 7-d cultivated human M $\phi$  in the absence of serum. At least 50–100 cells on triplicate coded preparations were viewed by electron microscopy to determine binding and/or ingestion. As anticipated, M $\phi$  were able to bind (Fig. 3 A and B) and ingest unopsonized and opsonized (not shown) zymosan particles. In contrast, PMN were able to bind and ingest only iC3b zymosan (Fig. 3 C and D). Examination of electron micrographs showed that 22% of PMN in these sections bound or ingested iC3b zymosan after incubation for 15 min at 37°C compared with 2% uptake of unopsonized zymosan ( $n = 200$ ). This method of analysis underestimates the actual percentage of PMN that take up particles.



**Figure 3.** Phagocytosis of unopsonized and iC3b-opsonized zymosan by human M $\phi$  and PMN, respectively. (A and B) Scanning electron micrographs. 7-d cultured human M $\phi$ -bound unopsonized zymosan (arrows). (A)  $\times 4,500$ . (B) An enlargement of central region of cell,  $\times 18,000$ . (C and D) Transmission electron micrographs. Freshly iso-

lated PMN ingested only iC3b-zymosan. (C)  $\times 9,900$ . (D)  $\times 15,000$ . PMN exposed to unopsonized zymosan bound or ingested very few particles. Note loose apposition between PMN plasma membrane and opsonized zymosan surface in D.

Phase-contrast microscopy confirmed that virtually all PMN could bind or ingest iC3b-zymosan, whereas uptake of unopsonized zymosan was <10%.

#### *Interaction of M $\phi$ and PMN in response to unopsonized zymosan*

In view of the foregoing observations we asked whether M $\phi$  were able to opsonize zymosan particles for recognition by PMN. We designed an experiment in which M $\phi$  were exposed briefly to unopsonized zymosan under serum-free conditions, before addition of PMN which by themselves would remain unreactive to unopsonized zymosan. We assayed respiratory burst induction as a measure of the PMN response because this was suitable for analysis at the single cell level (NBT reduction) and preliminary experiments with PMN in suspension confirmed that PMN produced copious amounts of O<sub>2</sub><sup>-</sup> when triggered with PMA or fresh serum-treated zymosan, but not in response to unopsonized zymosan (legend to Fig. 4). We chose 7-d cultured blood monocytes as the source of M $\phi$  in these cell collaboration experiments because prolonged adhesion results in loss of respiratory burst activity to background levels ([27, 28] Fig. 4 legend), while the ability to bind and ingest unopsonized zymosan via the CR3 and to secrete complement (5) is substantially retained. Furthermore, experiments with freshly isolated monocytes confirmed that these cells themselves release O<sub>2</sub><sup>-</sup> upon direct challenge with unopsonized zymosan (not shown) and were unsuitable for co-cultivation experiments. Finally, we found that isolated PMN were triggered to release O<sub>2</sub><sup>-</sup> upon initial contact with fresh glass coverslips (27). This could be prevented by using glass coverslips which had been coated by prior incubation in heat-inactivated human serum, as used for M $\phi$  cultivation (Fig. 4 legend).

We therefore incubated 7-d M $\phi$  with unopsonized zymosan for 10 min at 37°C, in the absence of serum, prior to addition of freshly isolated PMN. The PMN clustered around M $\phi$  containing zymosan and released large amounts of O<sub>2</sub><sup>-</sup> as determined by NBT reduction and cytochrome *c* reduction (Fig. 4 E and F and legend). When the interaction continued for longer than 15 min, the M $\phi$  monolayer was destroyed and there was also PMN death (not shown). Fig. 4 C and D show that in the absence of zymosan there was no specific M $\phi$ -PMN aggregation and very little O<sub>2</sub><sup>-</sup> release. These experiments indicated that M $\phi$  were able to provide opsonins and/or chemotactic products that enable PMN to respond to the presence of zymosan with a vigorous respiratory burst.

## Discussion

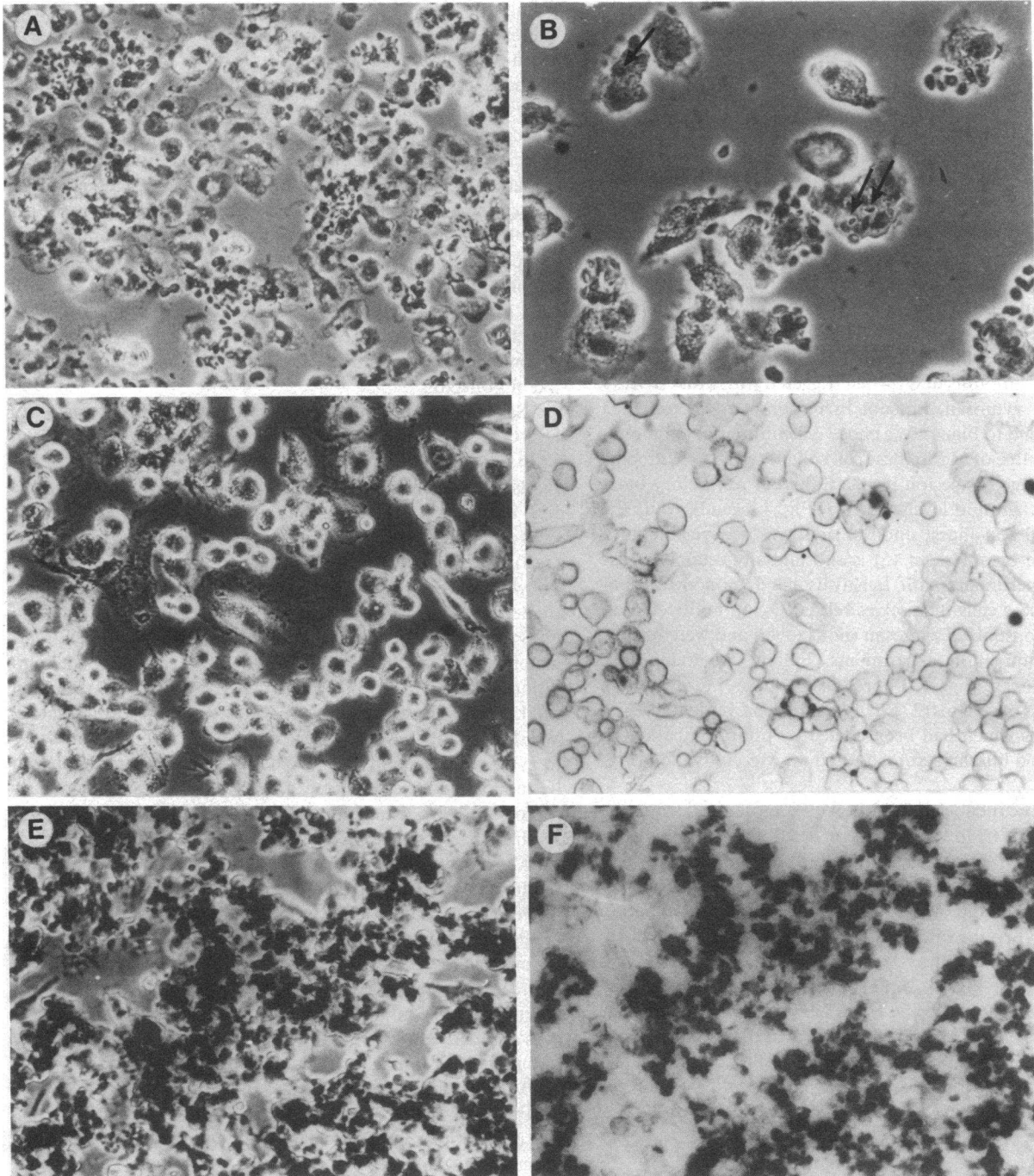
In this study we show that (a) zymosan particles incubated with M $\phi$  without serum accumulate iC3b on their surface; (b) monoclonal antibodies directed against the ligand binding site of CR3 inhibit zymosan uptake by M $\phi$ ; (c) M $\phi$  receptors for mannosyl residues and for iC3b that mediate zymosan uptake are distinct and can be independently modulated; (d) human PMN that express CR3 bind and ingest zymosan only after exogenous opsonization; (e) 7-d cultivated human M $\phi$  enable PMN to react with unopsonized zymosan in the absence of serum, with resultant release of O<sub>2</sub><sup>-</sup>.

We have provided further evidence indicating a role for M $\phi$ -derived alternative pathway components and the CR3 in local opsonization of zymosan. In addition to C3, we demonstrated factor H in monocyte-conditioned medium by biosynthetic la-

beling and eluted polypeptide chains of iC3b and C3b from zymosan particles incubated with monocytes in the absence of serum. The use of zymosan as a trap for covalently bound C3 provides a powerful direct probe to detect C3 and related molecules. The alternative pathway components found in monocyte/M $\phi$ -conditioned media represent only a small fraction of secreted protein and have been detected by sensitive functional assays or partial purification using affinity (1) or ion-exchange chromatography. Other proteins found in relatively large amounts after biosynthetic labeling and not thought to be complement components include a 95,000-mol wt polypeptide similar to a major gelatin-binding protein described by Vartio et al. (23). This molecule is distinct from fibronectin, which can also be secreted by human M $\phi$  (29), and there is no evidence that it is opsonic or able to modulate complement receptor function (30), like fibronectin.

Selective inhibition of zymosan uptake by Fab fragments of anti-C3 and by two antibodies that interfere with binding of iC3b ligands (M01, OKM10), but not by IB4 antibody, which binds to a different CR3 epitope, provides additional evidence that zymosan binds, at least in part, to the ligand binding site of M $\phi$  CR3 although other receptors are also likely to be involved (1, 8). The exact nature of the ligand-receptor interaction is not known, but inasmuch as C3 is a glycoprotein it has been postulated that CR3 might be a lectin-like receptor (31). Zymosan-CR3 interaction would therefore be explained by a direct interaction of a sugar shared with iC3b hence accounting for inhibition by the CR3 antibody. Our study makes this explanation unlikely in the case of monocyte/M $\phi$  interaction with zymosan. We show that CR3 and the lectin-like MFR, which contributes to zymosan uptake by M $\phi$  populations with this receptor, can be modulated independently. Modulation of complement receptors in RPM demonstrated here with anti-CR3 antibody resembles that observed with human monocytes (30), but differs from a previous report where substratum-bound C3 failed to modulate the CR3 in RPM (18). Successful modulation may be the result of a greater affinity of the monoclonal antibody than the ligand for the receptor. It is possible that the failure of RPM to ingest iC3b ligands is not a function of lack of mobility of the receptors, but reflects their state of nonactivation (32). In addition, soluble mannan does not inhibit EAiC3b rosetting nor does anti-CR3 antibody inhibit MFR activity (1). Together these results make it unlikely that mannose plays a role in the M $\phi$ -iC3b/CR3 interaction studied here. We do not know whether the two receptors recognize distinct ligands on the zymosan surface or a complex ligand such as the mannose-rich peptidoglycan found in cell walls of yeast, parasites, and some bacteria which may act as both an acceptor surface for initial complement deposition and express exposed mannose residues. Further deposition of complement could mask free mannosyl-residues so that uptake of serum-opsonized targets proceeds almost exclusively via CR3 (1, 8). Finally, exogenous (30, 33) or M $\phi$ -derived fibronectin (23) can influence phagocytic recognition by direct or indirect involvement of CR3. It is possible that iC3b is a major, but not exclusive ligand for the active site of CR3 in these complex interactions.

Although PMN and M $\phi$  share CR3 receptors, PMN, unlike M $\phi$ , did not ingest zymosan particles in the absence of an exogenous opsonin (cf. 9, 10), nor did unopsonized zymosan elicit a respiratory burst by PMN. It is possible that the CR3 and other lymphocyte function antigen (LFA) molecules in PMN (34, 35) also contribute directly to PMN adhesion to substrata or par-



**Figure 4.** Unopsonized zymosan triggers a respiratory burst by human PMN cocultivated with human M $\phi$ . NBT reduction shown in phase-contrast micrographs, except D and F, which are bright-field micrographs. O<sub>2</sub><sup>-</sup> release in parallel experiments performed in absence of NBT is given in brackets. (A) PMN cultivated 15 min on pretreated glass coverslips (see Methods) with NBT and zymosan (58 nmol O<sub>2</sub><sup>-</sup>/10 min). Note occasional clumps of extracellular zymosan; the vast majority of PMN are free. (B) 7-d cultured M $\phi$  with NBT and zymosan (20 nmol O<sub>2</sub><sup>-</sup>/10 min). Arrows show ingested zymosan particles which are not readily discerned at this magnification. (C-F) Freshly isolated PMN added to 7-d M $\phi$  in presence of NBT without

(C and D, 32 nmol O<sub>2</sub><sup>-</sup>/10 min) or with (E and F, 218 nmol O<sub>2</sub><sup>-</sup>/10 min) zymosan. Note the extensive aggregation of PMN and marked NBT reduction seen only in the presence of zymosan particles, which are obscured. Control experiments with PMA and serum-treated zymosan (STZ) showed O<sub>2</sub><sup>-</sup> release/10 min as follows: PMN alone + PMA 230, +STZ 182; 7-d M $\phi$  alone + PMA 44; PMN with M $\phi$  + PMA 268, +STZ 204. Background release on surface without trigger (20–60) subtracted. Results show average of duplicate values in a single assay, representative of more than three independent experiments.

ticulate substances, in that patients genetically deficient in surface expression of this family of molecules display complex defects in PMN adhesion, even in the absence of complement. However,

the ligands for other lymphocyte function antigens have not been identified nor can the defects be ascribed to CR3 dysfunction alone because at least three surface molecules share a com-



mon  $\beta$ -chain which is lacking in leukocytes of these patients (36). Further studies are also needed with monocytes from such patients.

We obtained evidence that  $M\phi$  can interact with zymosan to induce a respiratory burst in PMN. In co-cultivation experiments we noted that PMN are attracted to zymosan-bearing  $M\phi$ , aggregate, and degranulate with resultant destruction of macrophages as well as PMN, presumably a result of the vigorous respiratory burst. These reactions depended on the simultaneous presence of both cell types and zymosan and could be due to reaction of PMN with "opsonized" zymosan or with products of the  $M\phi$ -zymosan interaction. We have not identified the possible  $M\phi$  mediators involved, e.g., complement products C3a, C5a (37), and/or leukotrienes (38), nor have we determined whether the CR3 is able to trigger a respiratory burst in PMN (39, 40). However, these observations indicate that even non-activated  $M\phi$  can collaborate with PMN in host defense. Initial migration of PMN to an extravascular site may not necessarily be accompanied by increased efflux of high levels of all plasma complement components. Local production of complement and other potential chemotactic products and opsonins by tissue  $M\phi$  could serve not only to mediate destruction of pathogens by  $M\phi$  themselves, but also to recruit and activate PMN, and thus to initiate and amplify a local inflammatory response.

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