LOCAL OPSONIZATION BY SECRETED MACROPHAGE COMPLEMENT COMPONENTS

Role of Receptors for Complement in Uptake of Zymosan

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Circulating monocytes and tissue macrophages (Mφ) secrete complement proteins and have distinct receptors for some of these secreted products. Although hepatocytes and possibly epithelial cells are major sources of plasma complement (1), Mφ synthesize C1 subcomponents (2, 3), C4 (4, 5), C2 (5–7) C5 (8), C3 (2, 8) and all the components of the alternative pathway and its control proteins (8). Current evidence indicates that Mφ bear at least two distinct receptors for fragments of activated C3 (9, 10). The type one complement receptor, CR1 (205 kD) displays specificity for activated C3 (C3b) (11) and C4 (C4b) (12). CR1 interacts with iC3b, the product of C3b cleavage by factor I (9), although anti-CR1 antibodies that block C3b-dependent rosetting do not block iC3b-dependent rosetting (13, 14). The type three complement receptor, CR3, (170 and 95 kD) interacts with iC3b, and possibly with the further degradation product C3dg (9).

In order to investigate the physiological role of Mφ complement products we made use of monoclonal antibodies (ab) that are specific for CR1 (13) and that recognize epitopes associated with CR3 (15, 16). We found that deposition of Mφ-derived C3 on the acceptor surface of zymosan particles mediated binding and ingestion of zymosan via Mφ CR3. Zymosan uptake could also be mediated via mannosyl, fucosyl receptors (MFR, 17) in those Mφ populations that possess both CR3 and MFR activity (18). These studies indicate a role for Mφ complement in local opsonization and host defense.

Materials and Methods

Animals

Swiss outbred (PO) and CBAT6T6 and C57bl mice were bred at the Sir William Dunn School of Pathology, Oxford, and both sexes used at 20–30 g.

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Abbreviations used in this paper: ab, antibody; BCG, bacillus Calmette-Guérin; C3, third complement component; CR1, type 1 complement receptor; CR3, type 3 complement receptor; iC3b, C3b cleaved by factor I; FBS, fetal bovine serum; IM, Iscove’s medium; MFR, mannosyl, fucosyl receptor; Mφ, macrophages; O2, superoxide anion; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPM, thioglycollate-elicited mouse peritoneal Mφ.
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 use. 100 μg/ml kanamycin, 50 μg/ml streptomycin, and 50 μg/ml penicillin were added to media. Phosphate-buffered saline A and B were obtained from Oxoid Ltd., Basingstoke, England. Thioglycollate broth was bought from Difco Laboratories, Detroit, MI. Bacillus Calmette-Guérin (BCG) Trudeau Strain 1011 was obtained from the Trudeau Institute, Saranac Lake, NY. 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 in SDS-PAGE were all obtained from Sigma Chemical Co., St. Louis, MO. Carboxylated latex beads, diameter 0.86 μm, 0.25 meq CO₂/g (Dew Diagnostic, Indianapolis, IN) were labeled with [3H]tyramine in the presence of toluene sulfate (19). Cycloheximide was purchased from Calbiochem-Behring Corp., La Jolla, CA.

Antibodies

The rat anti-mouse hybridoma MI/70 (anti-Mac 1) and the ab used as tissue culture supernatant were gifts of Dr. T. Springer, Harvard Medical School, Boston, MA. This defines the CR3 on mouse Mø, polymorphonuclear leukocytes (PMN), and NK cells (20). MOI, a mouse anti-human ab, a gift from Dr. R. Todd, Harvard Medical School, Boston, MA inhibits binding and uptake of iC3b ligands and was used as an ascites (16). F4/80, a rat anti-mouse ab specific for mature mouse Mø (21) and 3C10, a mouse anti-human monocyte ab, a gift from Dr. W. Van Voorhis, The Rockefeller University, NY were both used as concentrated hybridoma supernatants (22). WMI, a mouse ab that defines an antigenic determinant on the human C3c fragment was used as an ascites (23). TO5, a mouse anti-human CR1 ab (13) was a gift of Dr. David Mason, Oxford, and was used as a tissue culture supernatant. All ab were used at saturating concentrations in all experiments.

Cells

Mouse macrophages. Thioglycollate-elicited Mø (TPM) were obtained 4–5 d after intraperitoneal injection of thioglycollate broth. BCG-activated peritoneal Mø (BCG-PM) were harvested 7–14 d after intraperitoneal infection with ∼1 × 10⁷ live organisms. Peritoneal cells were washed and resuspended in either IM or IM + 5% FBS and plated in 24-well tissue culture trays or on glass coverslips at 8 × 10⁵ or 2 × 10⁵ Mø per well, respectively. Adherent monolayers were prepared after 2–4 h incubation at 37°C in 5% CO₂ by washing twice with phosphate-buffered saline (PBS). J774, a murine Mø cell line, was maintained in spinner bottles at 37°C. Cells were obtained from T75 tissue culture flasks by vigorous agitation, and adhered for 2–4 h in 24-well tissue culture flasks before use.

Isolation of human monocytes. “Buffy coat” from 150 ml of freshly drawn human blood from laboratory personnel or from the John Radcliffe Hospital Blood Transfusion Service was diluted twofold with IM and mononuclear cells 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 cultivated in 24-well tissue culture trays at 37°C in 5% CO₂ for 45–60 min. An adherent monolayer was obtained by washing twice with PBS.

Culture of human monocytes. Purified monocytes were cultivated in 24-well tissue culture trays in IM + 20% heat-inactivated autologous serum at 37°C in 5% CO₂ for 7 d before assay. During this time cells became well spread and resembled mature Mø morphologically.

Ligands. A glycoconjugate of mannose-bovine serum albumin (MBSA) with 33–37 mol of sugar/mol protein was a gift from Dr. P. Stahl, Washington University, St. Louis, MO. This material was trace labeled with ¹²⁵I and used at 8 × 10⁵ cpm/ng. Zymosan was
boiled for 15 min in 5 ml PBS and pelleted by centrifugation. Zymosan was iodinated by a modification of the chloramine T method of Greenwood et al. (24) as described by Badwey et al. (25). Briefly, 250 mg of zymosan was resuspended in 5 ml PBS and 150 μl of 1 mg/ml chloramine T added with 1.5 mCi of Na\(^{125}\)I (Amersham, U.K.) at 4°C for 20 min. The reaction was terminated by the addition of 200 μg of potassium metabisulfite. The zymosan was washed five times with PBS, resuspended in 20 ml PBS, and dialyzed overnight against 500 ml of PBS containing 10 mM sodium iodide. After dialysis the zymosan was pelleted, washed, and stored in PBS at -20°C. ∼8 × 10⁵ zymosan particles in 10 μl (4,000 cpm/μl) were added, routinely, to each assay well.

Assays

**Uptake and binding of \(^{125}\)I-zymosan.** Mouse Mφ or human monocytes were preincubated with or without saturating amounts of anti CR3 ab or 1 mg/ml yeast mannan in IM for 15 min at 37°C in 5% CO₂. After a further 10 min incubation with \(^{125}\)I-zymosan (∼100 zymosan particles/cell), the cells were washed five times to remove free zymosan. To determine total binding and ingestion, cells were solubilized in 1 M NaOH and the cell-associated radioactivity measured in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). Ingestion was assayed as follows. Attached zymosan was removed by 15 min treatment with 2.5% wt/vol trypsin solution (Gibco-Biocult Ltd.) at 37°C and confirmed by phase contrast microscopy and staining with trypan blue. Extracellular zymosan takes up trypan blue, unlike ingested particles, thus providing a sensitive method to detect residual surface zymosan. Routinely, ab were washed out before addition of \(^{125}\)I-zymosan and the assay medium did not contain FBS. However, some assays were performed in the continuous presence of anti-CR3 ab and 5% HI FBS with no difference. Mannan was present continuously in all experiments. Degradation of \(^{125}\)I-zymosan could be detected after 60 min incubation with cells at 37°C by the appearance of trichloracetic acid-soluble radioactivity in the medium. Control cultures without cells did not release any acid-soluble radioactivity. Results were expressed as cpm/μg cell protein, detected by the Lowry method (26), and as a percentage inhibition of uptake or of total cell-associated \(^{125}\)I-zymosan. Nonspecific binding and uptake were determined by assaying L929 fibroblasts, which lack mannose/fucose and complement receptors, (R. A. B. Ezekowitz and J. Cardosa, unpublished), under conditions similar to Mφ monolayers. These cells bound 10–20% of \(^{125}\)I-zymosan when compared with matched Mφ populations.

**Mannose-specific endocytosis.** Uptake was measured using saturating amounts of trace-labeled mannose-BSA as described (27), and was inhibited by mannose-rich yeast mannan (1 mg/ml) or ribonuclease B (1 mg/ml). Results were expressed as ng ligand/μg cell protein.

**Antigens.** Binding of MI/70 to Mφ that had been fixed for 10 min in 0.25% wt/vol glutaraldehyde was detected as described (18). Binding of MOI to human monocytes was detected by using a radiiodinated F(ab')₂ rabbit anti-mouse ab, a gift from Dr. A. F. Williams, University of Oxford. Both first and second stage ab were used at saturation and the number of sites per cell determined (28).

**Single cell analysis.** Coverslip preparations of 2–4 h adherent Mφ were incubated with 100 unlabeled zymosan particles/cell with or without anti-CR3 ab and/or yeast mannan, 1 mg/ml in IM. Preparations were washed five times, treated with 2.5% wt/vol trypsin for 15 min at 37°C to remove attached zymosan, fixed in 0.25% wt/vol glutaraldehyde, and ingestion-scored under phase contrast microscopy. 300 cells were examined in duplicate preparations.

**Preparation of C3b-zymosan and iC3b-zymosan.** C3b-zymosan was prepared by incubating fresh PO mouse serum with 20 mg of boiled zymosan for 20 min at 37°C. This was incubated with 0.5% wt/vol sodium dodecyl sulphate (SDS) at 37°C for 15 min and washed five times in PBS to remove adsorbed serum protein (29). To prepare iC3b zymosan the C3b zymosan was incubated with isolated Factor I from human plasma and purified factor H in 10 mmol KPO₄⁻, pH 7.4, for 30 min at 37°C. The ligands were eluted by further incubation with 1 M hydroxylamine, pH 9.0, and 0.5% wt/vol SDS (29), which removed C3b and iC3b as determined by 9% SDS-polyacrylamide slab gel...
electrophoresis (results not shown). Mouse iC3b resembled human iC3b (30). Binding of anti-CR3 ab to these ligands and untreated boiled zymosan was tested by indirect binding assay.

Isolation of C3. C3 was isolated from human plasma and, where indicated, converted to C3b or labeled with \(^{125}\text{I}\) to a specific activity of 1.0–1.8 \(\times 10^6\) cpm/\(\mu\)g as described (31, 32). Factors I and H were isolated as indicated (33).

Purification of C3 from culture supernatants. Human monocytes were incubated with \(^{[35}\text{S}]\text{methionine (Amersham, U.K.)}, 750 \mu\)Ci/5 ml methionine-free MEM, supplemented with 1% dialyzed FBS, for 4 h. Unlabeled human C3 (50 \(\mu\)g) was added as carrier and supernatants (0.5–10.0 ml) were precleared by incubation (1.5 h, 4\(^{\circ}\)C) with 1.0–5.0 ml (packed volume) of nonspecific rabbit IgG bound to Sepharose 4B (Sepharose-IgG) in 250 mM NaCl-20 mM EDTA-0.5 mM methionine, pH 7.4. Culture supernatants were then incubated (12 h, 4\(^{\circ}\)C) with 1.0–5.0 ml (packed volume) of rabbit-IgG anti-human C3 bound to Sepharose 4B (Sepharose-anti-C3). The Sepharose-anti-C3 had a binding capacity of 120 \(\mu\)g C3/ml packed Sepharose. Sepharose-anti-C3 and Sepharose-IgG were subsequently washed six times with 2 vol of the buffer noted above. Non-covalently-bound protein was removed by incubation (30 min, 4\(^{\circ}\)C) in 4 vol of 0.1 M glycine-HCl, pH 2.2. Eluted material was lyophilized and examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography (34, 35).

To investigate whether C3 in culture supernatants is in the native (active) form, the C3, still bound to Sepharose-anti-C3, was resuspended in water at pH 9.0, and incubated at 100\(^{\circ}\)C for 15 min to induce autolytic cleavage of native C3 (32). C3 was then eluted as above and examined by SDS-PAGE and autoradiography.

Detection of C3 convertase components in monocyte supernatants. Culture supernatants (300 \(\mu\)l of 50-fold concentrated material) from human monocytes cultured in serum-free medium were incubated at 37\(^{\circ}\)C with \(^{125}\text{I}\)-labeled C3 (30 \(\mu\)l; 18 \(\mu\)g) and 300 \(\mu\)l of a suspension of zymosan (10 mg/ml in 2.5 mM sodium veronal-0.15 mM CaCl\(_2\)-0.5 mM MgCl\(_2\)-72.5 mM NaCl-2.5% wt/vol d-glucose-0.1% wt/vol gelatin (DGVB\(^+\) buffer)). Portions were withdrawn at intervals (0–60 min) and the zymosan washed exhaustively with DGVB\(^+\), water, 1 M NaCl, and 0.1% wt/vol SDS. The \(^{125}\text{I}\) radioactivity associated with zymosan was determined and the rate of deposition of activated \(^{125}\text{I}\)-labeled C3 onto zymosan was calculated. Dilutions of fresh normal human serum were used as positive controls. This assay is mainly a measure of factor B + D activity, although classical pathway involvement is not specifically excluded.

Detection of factor I plus cofactor activity in monocyte supernatants. The rate of breakdown of \(^{125}\text{I}\)-labeled C3b to the defined product iC3b in the presence of serum-free monocyte culture supernatants concentrated 50-fold was determined by SDS-PAGE and autoradiography (30, 33). This assay measures factor I plus factor I-cofactor activity. Mixtures of isolated factors H + I were used as positive controls.

Assay for alternative pathway activation in human serum. The capacity of mannan to activate the alternative pathway was measured as described by Riches and Stanworth (36).

Inhibition of covalent binding of C3. The covalent binding of activated C3 was inhibited by the use of sodium salicyl hydroxamate as described by Sim et al. (31).

Detection of M6 C3 on zymosan under assay conditions. Direct evidence that M6-secreted C3 was trapped on zymosan under assay conditions was obtained by preincubating a monocyte monolayer, 5 \(\times\) 10\(^5\) cells/35-mm tissue culture dish, with an inhibitor cocktail of MOI, mannan, and TO 5 for 10 min at 37\(^{\circ}\)C to prevent zymosan uptake. Unlabeled zymosan, 100 particles/cell, was then added for 10 min and collected, pelleted in a microfuge and bound C3 detected by indirect binding assay using WMI as first-stage ab and radiolabeled rabbit anti-mouse second stage ab. The number of C3 molecules bound per zymosan particle was calculated by the method described by Mason and Williams (28).

Superoxide assay. Superoxide anion was assayed after addition of 10 \(\mu\)g of zymosan (37). Macrophage monolayers were preincubated with M1/70 and/or ribonuclease B, 1 mg/ml before addition of 10 \(\mu\)g of zymosan or 20 ng of PMA. Results were expressed as \(O_2^-\) released/\(\mu\)g cell protein/15 min. Superoxide dismutase, 25 \(\mu\)g/ml inhibited >80%
of cytochrome c reduction. Zymosan in the reaction mixture was routinely removed by pelleting in a microfuge for 90 s.

Results

Role of CR3 and MFR in Binding and Uptake of 125I-Zymosan by Human Monocytes and Macrophages

To examine the role of specific cellular receptors in binding of unopsonized zymosan, human blood monocytes (1 h) and culture-derived Mo (7 d) were incubated with 125I-zymosan in the presence or absence of Mo1 and mannan, inhibitors of CR3 and MFR activity, respectively. Receptor expression was measured by specific uptake of 125I-mannose BSA and indirect binding assay of Mo1 antigens. Table I shows that freshly isolated human monocytes bind zymosan mainly by the CR3. These cells express the CR3 antigen MO1 (1.8 × 105 molecules bound per cell) and preincubation with MO1 inhibited binding and uptake of radiolabeled zymosan by 78%. Lack of inhibition by mannan is consistent with the low levels of MFR activity expressed by this population (Table I; T. Mokoena and S. Gordon, unpublished). Most of the monocyte-associated zymosan was bound, rather than ingested. 85% of 125I-zymosan could be removed from the monocyte surface by trypsin treatment; single cell analysis, not shown, revealed that although almost all 1 h adherent monocytes bound zymosan, <80% ingested particles. Where ingestion occurred, only one to two particles/cell were taken up. This result is consistent with observations that freshly isolated human monocytes bind rather than ingest opsonized sheep erythrocytes via both C3b and iC3b receptors (14).

TABLE 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cell associated 125I-zymosan (cpm/μg cell protein)</th>
<th>Percent inhibition of total binding and uptake after treatment with:</th>
<th>Receptors</th>
<th>MO1 Antigens (molecules 2nd stage ab bound per cell)</th>
<th>MFR (ng uptake 125I-mannose BSA/μg protein/20 min at 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Ingestion MO1 Mannan MO1 + mannann</td>
<td></td>
<td></td>
<td>(molecules 2nd stage ab bound per cell)</td>
<td>(ng uptake 125I-mannose BSA/μg protein/20 min at 37°C)</td>
</tr>
<tr>
<td>Human monocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>42 6 78 4 80 1.8 0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>96 58 43 40 71 2.4 2.40</td>
<td></td>
<td></td>
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</tbody>
</table>

Human monocytes were adhered for 1 h or cultivated for 7 d in 24-well tissue culture trays before assay of zymosan uptake and receptor expression. Monolayers were incubated in the presence or absence of inhibitors for 15 min before addition of 125I-zymosan for 10 min at 37°C. The total cell-associated zymosan represents binding and ingestion; ingestion was determined after 15 min treatment with trypsin. Results show one experiment done in triplicate, SD <10%, representative of five independent experiments. Cell protein was 40 ± 10 μg (1 h), 68 ± 6 μg (7 d).
In contrast, human Mφ obtained after 7 d cultivation in autologous serum expressed MFR and CR3 activity and both receptors mediated uptake of 125I-zymosan. (Table I; T. Mokoena and S. Gordon, unpublished). Total uptake of 125I-zymosan by these Mφ was greater than by monocytes. MO1 and mannan now inhibited uptake to a similar extent, 40% each, and this effect was additive, 71%. In the absence of inhibitors more than half of zymosan bound by 7 d Mφ was ingested and single cell analysis after trypsin treatment (not shown) indicated relatively uniform ingestion, up to three or more particles/cell, after treatment with MO1 or mannan.

Control experiments established that MO1 did not bind directly to zymosan, unopsonized or coated with C3b or iC3b. Binding of 125I-zymosan to monocytes was not inhibited (<20%) by an anti-CR1 monoclonal ab TO 5 or by an unrelated anti-monocyte ab, 3C10. We concluded that the CR3 mediated recognition of zymosan, either alone, as in monocytes that express no MFR activity, or in combination with MFR, in culture-derived Mφ. We next examined zymosan uptake by other Mφ populations that express CR3 and variable levels of MFR.

**Effect of M1/70 and Mannan on Uptake of 125I-Zymosan by Mouse Mφ**

BCG-activated peritoneal Mφ and J774 cells are able to ingest unopsonized zymosan while expressing low or undetectable levels of MFR, respectively (Table II). Both these Mφ populations express the CR3 antigen detected by M1/70. Thioglycollate-elicited peritoneal Mφ, which ingest zymosan more vigorously, express both CR3 and MFR activity. The effects of M1/70 and mannan on uptake of 125I-zymosan by these mouse Mφ are also shown (Table II). Uptake by BCG-PM and J774 Mφ was inhibited 88 and 90%, respectively by M1/70, indicating that ingestion of zymosan was almost entirely via CR3. Zymosan uptake by TPM was partially inhibited by M1/70 (49%) and mannan (30%) when used separately and these effects were additive (70%). Similar effects by both inhibitors were noted with resident peritoneal Mφ (not shown).

Further control experiments were performed to characterize the effects of

### Table II

**Effects of M1/70 and Mannan on Uptake of 125I-Zymosan by Mouse Macrophages**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ingestion of 125I-zymosan (cpm/μg protein)</th>
<th>Percent inhibition of uptake after treatment with:</th>
<th>Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mac-1 antigens (molecules 2nd stage ab × 10^3/cell)</td>
<td>MFR (ng uptake 125I-mannose BSA/μg protein/20 min at 37°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1/70 Mannan M1/70 + mannan</td>
<td></td>
</tr>
<tr>
<td>BCG-PM</td>
<td>87</td>
<td>88 8* 90</td>
<td>5.0 0.63</td>
</tr>
<tr>
<td>J774</td>
<td>92</td>
<td>90 4 92</td>
<td>1.6 0.02</td>
</tr>
<tr>
<td>TPM</td>
<td>205</td>
<td>49 30 70</td>
<td>6.1 2.1</td>
</tr>
</tbody>
</table>

Macrophages were adhered for 2 h before assay. Cells were preincubated with inhibitors for 15 min before addition of 125I-zymosan for 10 min at 37°C. Cell protein in μg was: BCG, 40 ± 8; J774, 43 ± 6; TPM, 58 ± 5, respectively. Attached zymosan was removed by 15 min trypsin treatment. Results of 1 experiment representative of 10 independent experiments, SD ≤ 10%.

* Inhibition by mannan varied between 5 and 30% depending on experimental conditions (see Table IV and Fig. 1).
OPSONIZATION BY MACROPHAGE COMPLEMENT

Each inhibitor. M1/70 did not inhibit uptake of 125I-zymosan by human monocytes, to which the ab binds with less affinity than to murine Mφ. All assays were routinely performed in the absence of serum although similar results were obtained in the presence of 5% H1 FBS. Mannan inhibition required the continuous presence of the yeast wall product throughout the assay, whereas the anti-CR3 ab was usually washed out after 15 min preincubation. Mannan itself did not significantly activate the alternative pathway in serum. Mannan did not inhibit binding of M1/70 nor did M1/70 influence uptake of 125I-mannose-BSA. The uptake of 3H-latex beads, particles that do not activate the alternative pathway, was unaffected by mannan or anti-CR3 ab. M1/70 inhibited uptake of preformed iC3b 125I-zymosan to a greater extent than unopsonized zymosan although inhibition was not >80%. F4/80, a rat anti-mouse Mφ ab, did not affect zymosan uptake. Assays were routinely performed for 10 min at 37°C and results shown were highly reproducible.

Kinetics of inhibition

TPM and BCG-PM were incubated in serum-free medium with 100 zymosan particles per cell for varying periods at 37°C, in the presence of M1/70 and/or mannan. Uptake of 125I-zymosan was linear for 20 min and was greater for TPM (Fig. 1 B and D). Acid-soluble radioactivity did not appear in the supernatant of
either cell population before 50 min, so the plateau observed after 20 min was not due to degradation. The extent of inhibition by M1/70 varied markedly with time, but was relatively constant for mannan in both populations. With BCG-PM (Fig. 1A) inhibition of zymosan uptake by M1/70 was maximal (70%) at 5–10 min and then decreased rapidly by 20–30 min. Mannan inhibited 15% of uptake throughout this period, whether used alone or in combination. With TPM (Fig. 1C), inhibition by M1/70 was again maximal at 10 min (50%) and decreased rapidly thereafter. Inhibition by mannan was stable and was relatively greater (60%), as expected for this population. Both inhibitors together inhibited a maximum of 80%. These results confirmed the earlier findings that uptake of zymosan over short periods was mediated mainly by CR3 in BCG-PM, and by both CR3 and MFR in TPM, and that the inhibitors were additive. However, the CR3 contribution was short-lived.

Single cell analysis. To identify the cells that had taken up zymosan and to detect any possible heterogeneity, we next examined adherent populations by phase contrast microscopy. Table III shows that M1/70 reduced the number of zymosan particles/cell and the number of cells labeled in all Mφ examined. Only TPM, which ingested more particles per cell, showed a significant, partial inhibition by mannan, alone or in combination with M1/70. The results show mostly ingestion. Extracellular zymosan could be stained with trypan blue, and after trypsin treatment <10% of cells had attached particles. Previous autoradiographic studies revealed a similar uniform expression of Mac 1 and MFR by primary mouse Mφ populations (27).

Biosynthesis and secretion of alternative pathway complement proteins by human monocytes. Previous studies have shown that mononuclear phagocytes can synthesize and secrete all six alternative pathway proteins (8). It was important to demonstrate that under assay conditions the formation of iC3b-zymosan, the ligand recognized by CR3, was due to Mφ-derived complement proteins. These

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percent Mφ labeled after treatment with:</th>
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<tbody>
<tr>
<td></td>
<td>None</td>
<td>M1/70</td>
</tr>
<tr>
<td>TPM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>86</td>
<td>54</td>
</tr>
<tr>
<td>B</td>
<td>15</td>
<td>46</td>
</tr>
<tr>
<td>BCG PM</td>
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<tr>
<td>A</td>
<td>25</td>
<td>5</td>
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<td>B</td>
<td>75</td>
<td>80</td>
</tr>
<tr>
<td>J774</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>72</td>
<td>74</td>
</tr>
</tbody>
</table>

Table III

Single Cell Analysis of Effects of M1/70 and Mannan on Uptake of Zymosan by Mouse Macrophages

Macrophages were cultured for 2 h in 1M on glass coverslips and incubated with M1/70 and/or mannann for 15 min, washed, and ~100 unlabeled zymosan particles/cell added for 10 min at 37°C. Preparations were washed five times, treated with trypsin for 15 min to remove attached zymosan, then fixed in 0.25% glutaraldehyde and scored under phase contrast microscopy. 300 cells were counted in duplicate preparations. A and B represent percent cells with >5 or <5 particles/cell, respectively. SD < 15%.
experiments were done with human monocytes to facilitate detection of specific complement components.

**Biosynthesis of C3.** Freshly isolated human monocytes were cultured with [35S]methionine in the absence of zymosan and C3 isolated by affinity chromatography. Fig. 2 shows that labeled C3 was present in monocyte-conditioned medium in a native ("active") form. The C3 α and β chains are visible in track A (cf. standard track 1). Two bands at 76,000 and 43,000 mol wt are also visible in track A. These are characteristic of cleavage of inactive C3 (i.e., C3 in which the thiolester is hydrolyzed, but the polypeptide chain is intact) by factors H and I (30). This cleavage product is shown in standard track 2. In track B, where C3 has been treated to induce autolytic cleavage, two additional bands, characteristic of autolytic cleavage of C3, are visible at 74,000 and 46,000 apparent mol wt (cf. standard track 3). Since autolytic cleavage occurs only with active C3 (32) this shows clearly that a large proportion of the 35S-labeled C3 in the culture supernatant is active.

Evidence that new protein synthesis is also required for uptake of zymosan by mouse Mφ via CR3 but not MFR is given in Table IV. Treatment of peritoneal Mφ with cycloheximide but no inhibitor reduced the uptake of 125I-zymosan to an extent comparable to inhibition by M1/70 alone. Addition of cycloheximide and M1/70 inhibited to an extent similar to that of M1/70 alone. Addition of mannan augmented inhibition by cycloheximide to a greater extent in TPM than in BCG-PM, compatible with reduced expression of MFR by BCG-PM. Decreased uptake of zymosan via CR3 cannot be accounted for by a decrease in surface receptor expression, which is unaltered (<20% change) by cycloheximide treatment (Table IV).

**Detection of C3 convertase components in monocyte supernatants.** Serum free monocyte culture supernatants were found to stimulate activation and deposition of 125I-labeled C3 onto zymosan (Table V A). Rates of deposition observed were 1–3% of the rate observed with normal human serum. This demonstrates the

![Figure 2. Biosynthetic labeling of C3 by human monocytes. Tracks A–C, autoradiographs obtained after SDS-PAGE. (A), material eluted from Sepharose-anti-C3; (B), material heat-denatured to induce autolytic cleavage before elution from Sepharose-anti-C3; (C), total monocyte culture supernatants. Standard tracks 1–4 were stained with Coomassie Blue. Track 1, C3; track 2, inactive C3 partially cleaved by factors H and I; track 3, "active" C3 showing partial autolytic cleavage; track 4, molecular weight standards, myosin, C3b, catalase, ovalbumin, and carboxypeptidase A. Although H and I activity is detectable in serum-free monocyte culture supernatant, the cleavage seen here may be attributable to bovine H and I from fetal calf serum which also act on human C3b (50). Additional minor bands at about 100,000, 60,000, and 50,000 apparent mol wt are visible in track A, but these are not C3-derived.](image-url)
TABLE IV
Effect of Cycloheximide on Uptake of $^{125}$I-Zymosan via CR3 and MFR by Mouse Macrophages

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>Uptake [cpm (percent inhibition)] of $^{125}$I-zymosan</th>
<th>Receptors</th>
<th>MFR (ng uptake $^{125}$I-mannose BSA/µg protein/20 min at 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control M1/70 Mannan</td>
<td>M1/70 Ag (molecules 2nd stage ab × 10^5/cell)</td>
<td></td>
</tr>
<tr>
<td>BCG-PM</td>
<td>None</td>
<td>2,680 1,240 (54) 1,809 (32)</td>
<td>5.1 0.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cycloheximide</td>
<td>840 (69) 910 (66) 607 (78)</td>
<td>4.3 0.52</td>
<td></td>
</tr>
<tr>
<td>TPM</td>
<td>None</td>
<td>5,009 2,810 (46) 1,892 (62)</td>
<td>6.8 2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cycloheximide</td>
<td>2,516 (49) 2,340 (52) 1,550 (69)</td>
<td>5.4 2.0</td>
<td></td>
</tr>
</tbody>
</table>

5 × 10^5 1 h adherent peritoneal Mφ were preincubated for 2 h with 10 µg/ml cycloheximide at 37°C and maintained in its presence throughout the assay. Some wells were preincubated with inhibitors as described in Tables I and II. Results are expressed as cpm or percent inhibition of control in the absence of cycloheximide. Results shown are one experiment performed in triplicate (SD < 6%) representative of five independent experiments. Inhibition by mannan of uptake by BCG-PM varied (cf. Table II). 10 µg/ml cycloheximide had no effect on cell viability (>95% excluded trypan blue) and reduced plasminogen activator secretion by both TPM and BCG by 80 ± 15%, this effect was fully reversible.

TABLE V
Rates of C3 Deposition and C3b Degradation in the Presence of Human Monocyte Culture Supernatants

A. C3 deposition on zymosan

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate of C3b deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte supernatant</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>Control with undiluted human serum</td>
<td>1,600</td>
</tr>
</tbody>
</table>

B. C3b breakdown to iC3b

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate of iC3b formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte supernatant</td>
<td>1.34 ± 0.56 × 10^3</td>
</tr>
<tr>
<td>Control with 400 ng/ml H</td>
<td>3.6 × 10^3</td>
</tr>
<tr>
<td>80 ng/ml I</td>
<td></td>
</tr>
</tbody>
</table>

C3 convertase components were detected by measuring the rate of C3 deposition on zymosan. Factor I plus cofactor activity was assessed by measuring the rate of iC3b formation from C3b, as described in Materials and Methods. The rate of C3b deposition (A) was linear over 15–20 min, with a stable background, and the minimum cumulative deposition detectable was ~6 pg/min. For iC3b formation, the minimum detectable rate was ~80 pg/min.

presence of C3 convertase components in the monocyte supernatant. Similarly concentrated serum-free monocyte supernatants were shown to degrade soluble $^{125}$I-labeled C3b to the defined product iC3b (Table VB). This indicates the presence of factor I plus a factor I cofactor, most likely factor H.

Evidence for C3 binding to zymosan under assay conditions. In order to establish
OPSONIZATION BY MACROPHAGE COMPLEMENT

Table VI

<table>
<thead>
<tr>
<th>Cells</th>
<th>Inhibition [nmol O₂/mg protein/15 min (percent inhibition)] after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>BCG-PM + PMA (20 ng/ml)</td>
<td>128.8 ± 8</td>
</tr>
<tr>
<td>+ zymosan</td>
<td>76 ± 16</td>
</tr>
<tr>
<td>TPM + PMA</td>
<td>90.8 ± 6</td>
</tr>
<tr>
<td>+ zymosan</td>
<td>63.8 ± 12</td>
</tr>
</tbody>
</table>

8 × 10⁵ cells were cultivated in IM + 5% FBS for 2 h and washed three times with HBS. Cells were preincubated with inhibitors (see legend to Table I and II). Ribonuclease B (1 mg/ml) was used to inhibit MFR since it does not scavenge O₂⁻, unlike mannan (37). MI/70 was washed out before addition of reaction mixture. The percentage inhibition was calculated, as follows: 100 – (Inhibition blank)/(Stimulation blank). Blank values for BCG-PM and TPM were 10 and 8 nmol O₂/mg cell protein, respectively. Experiments show mean ± SD of data from duplicates in one experiment, representative of five independent experiments. The results obtained were 60% of values obtained when assay time was 60 min. MI/70 or ribonuclease B did not cause O₂⁻ release.

that transfer of Mφ complement components onto zymosan occurred under the conditions of assay, unlabeled zymosan was incubated for 10 min with a human monocyte monolayer in the presence of a cocktail of inhibitors to prevent uptake. The zymosan was then removed and the presence of C3 demonstrated by a trace indirect binding assay using an anti-C3 ab, WMI (23). Specific binding to zymosan incubated in the presence of monocytes was ~4.3 × 10³ C3 molecules/zymosan particle. This ab did not detect significant amounts of C3 antigen on the surface of monocytes prepared as for assays. Further evidence of a role for C3 in zymosan uptake was obtained by addition of the nucleophile sodium salicyl hydroxamate (1–5 mM), which inhibited uptake by BCG-PM and J774 Mφ 45–60%, comparable to that achieved by MI/70, without affecting cell viability. The hydroxamate is a potent inhibitor of the covalent binding reaction of activated C3 (31).

Role of CR3 in superoxide secretion. During phagocytosis or upon appropriate stimulation of the plasma membrane by surface active agents like phorbol esters, immune complexes, and both opsonized and unopsonized zymosan, activated Mφ release substantial amounts of O₂⁻ and H₂O₂ (37). Table VI shows that although MI/70 inhibited zymosan-triggered release of O₂⁻ by BCG-PM, PMA could still trigger superoxide release. In contrast, inhibition of zymosan-triggered superoxide release by TPM was dependent upon both MFR and CR3 activity.

Discussion

Zymosan is widely used to stimulate a respiratory burst in phagocytic cells (37) and to trigger release of arachidonate metabolites (38) and other secretory products (39). Earlier studies showed that zymosan that had not been opsonized by incubation with fresh serum retained much of its biological activity and that the MFR could mediate uptake of these particles (17, 37). However, Mφ populations that lack MFR activity, e.g., human monocytes and J774 cells (40), nevertheless bind or ingest unopsonized zymosan. The present studies show that the uptake of "unopsonized" zymosan by mononuclear phagocytes can be mediated by receptors for complement (CR3) as well as carbohydrate (MFR) and
that these receptors act independently or in concert. We have demonstrated that C3-derived components are present even in the absence of exogenous complement and have shown that the MΦ themselves can generate all the alternative pathway components required for opsonization. Our studies provide the first evidence that secretion of complement components by MΦ plays a direct role in regulating MΦ recognition and effector functions. Similar feedback mechanisms could link other MΦ secretory and endocytic pathways, e.g., via intermediates such as α2-macroglobulin (41, 42).

Monoclonal ab and other ligands with defined specificity were used to evaluate the role of distinct MΦ receptors in uptake of zymosan. The MΦ CR3 was implicated by the use of two independent monoclonal ab. M1/70 specifically prevents binding to mouse MΦ of iC3b-coated but not C3b-coated erythrocytes or E1gG (15) and inhibits immune enhancement of flavivirus replication mediated by complement, but not IgG (43). MO1 immunoprecipitates the human analogue of Mac-1 and also blocks binding of EiC3b rosettes to normal human monocytes (16). We have provided further evidence for selective action by these ab. Antibodies against CR1 or other monocyte MΦ ag were inactive, the uptake of latex was not affected, and L cell fibroblasts, which do not express Mac-1, failed to take up zymosan. Inhibition by mannan was not due to consumption of complement, and mannan and ribonuclease B, known inhibitors of MΦ MFR activity (44), showed comparable inhibitory effects.

All MΦ populations examined, including human and mouse cells, primary inflammatory, and activated MΦ and an MΦ line expressed Mac-1 or MO1 antigens and showed substantial inhibition of endocytosis, usually 50% by these ab. Inhibition of zymosan uptake by mannan reflected the level of MFR activity and varied from undetectable (human monocytes, J774) to 50% (human culture-derived MΦ, TPM); BCG-activated MΦ were intermediate. The contribution of each receptor was characteristic for each MΦ population and varied independent with cell type and conditions of assay. Inhibitors directed against each receptor were additive until a maximum level, normally no greater than 80–90%, was reached. Although it is conceivable that a third receptor is involved in uptake of zymosan, our studies with another ab T05 that has been shown to inhibit C3b-dependent rosetting to B lymphocytes (13) argue against a major role for CR1. Single cell analysis showed that inhibition was uniform within populations. Further studies are needed to establish whether dual recognition by both CR3 and MFR operates for each cell and each particle.

Inhibition by the anti-CR3 ab implies that ligands derived from C3 such as iC3b are generated on the surface of the zymosan particles when these are added to MΦ cultures. Serum was not required to demonstrate this phenomenon and no adsorbed C3 could be detected on the surface of freshly isolated human monocytes. Direct evidence was obtained that MΦ themselves synthesize and secrete active C3 under similar experimental conditions, as reported also by others (45). Furthermore, a requirement for ongoing protein synthesis in uptake via CR3, but not MFR, was demonstrated with cycloheximide which did not substantially alter receptor expression. Other unpublished observations are com-
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patible with a requirement for continuing complement synthesis/secretion. Zy-mosan binds poorly to Mφ at 4°C, although receptor function might then be affected. In the present report we showed further that C3 antigen could be detected on zymosan after exposure to human monocytes and that sodium salicyl hydroxamate inhibited uptake of zymosan, presumably by inhibiting formation of a C3-zymosan ester bond (29, 31).

Other complement system activities were detected in serum-free human monocyte supernatants. The results which showed that such supernatants stimulated deposition of 125I-labeled C3 onto zymosan can be most simply interpreted by assuming that the monocytes synthesize factors B and D, and possibly P, and can therefore assemble the alternative pathway C3b Bb(P) convertase on the zymosan surface. Whaley (8) has demonstrated the presence of functionally active B, D, and P in short-term (<1 d) human monocyte cultures, although Cohen (46) suggested that significant secretion of factor B does not occur until monocytes have been cultured for >3 d. Specific cleavage of 125I-labeled C3b to iC3b in the presence of serum-free monocyte culture supernatant demonstrates the presence of factors H and I in the supernatant. Whaley (8) has demonstrated secretion of active factor I in short-term monocyte cultures. Factor H activity was not detected in Whaley's study, although secretion of factor H antigen was demonstrated. The factor H assay used by Whaley was likely to be less sensitive than that described here. The complement receptor CR1 has the same factor I cofactor activity as H, but has not been reliably detected in soluble form. It is therefore unlikely that the cofactor activity in the supernatants could be attributed to CR1 rather than to H. All the alternative pathway components (47) can thus be generated simultaneously in serum-free Mφ cultures, as demonstrated recently by Brade (48).

In light of these considerations it is reasonable to conclude that the locally synthesized Mφ alternative pathway proteins opsonize zymosan particles which, in turn, bind to Mφ CR3. Several interesting features of this reaction should be noted. Kinetic experiments revealed that the process is fully developed within 5-10 min, but transient. Rapid decay of CR3 involvement could be due to complement lability or consumption of complement components by an excess of zymosan. Since C3 and other components could be detected in supernatants in the absence of zymosan it is likely that complement secretion is constitutive and not dependent on a particulate stimulus. Expression of the CR3 antigen Mac-1 is also constitutive since it appears early during Mφ differentiation (S. Hirsch and S. Gordon, unpublished observations) and remains at a relatively stable level during Mφ activation unlike several other plasma membrane determinants (27). However, function of the CR3 does vary markedly during cell maturity and in response to inflammatory (49), immune (50), and pharmacologic stimuli (14). In the present studies locally opsonized zymosan could trigger binding, ingestion, and/or release of O2 via CR3 as well as the MFR. Unpublished experiments with freshly isolated human blood monocytes that lack mannose-receptor activity indicate that the CR3 by itself can mediate these responses.

Our studies indicate that complement components synthesized by Mφ at
extravascular sites could play an important role in opsonization and lysis of cellular and microbial targets that activate the alternative pathway. In addition, complement-derived complexes, proteinases, and peptides could regulate the functions of macrophages themselves, as well as other cells of the immune system (51, 52).

Summary

We have examined the role of macrophage (Mø) plasma membrane receptors for the cleaved third complement component (iC3b; CR3) and mannosyl, fucosyl terminated glycoproteins (MFR) in uptake of unopsonized zymosan. Monoclonal antibodies against CR3, M1/70 (Mac-1) and MO1, each inhibited ~50% of uptake of 125I-zymosan by murine and human Mø, respectively. Yeast mannan inhibited 0–50% of zymosan uptake in various Mø, in parallel with their expression of MFR. We demonstrated that Mø were the source of C3 in our assay and that the activity of other components of the complement system, namely a C3 convertase, factor I, and a factor I cofactor were also present in serum-free cultures of human monocytes. Macrophage C3 was deposited rapidly, within 10 min, on the zymosan particles and mediated binding, ingestion, and stimulation of superoxide release in BCG-activated and thioglycollate-elicited peritoneal Mø via CR3. Local secretion of complement proteins by Mø themselves can therefore opsonize pathogens and cells able to activate the alternative pathway and effect their destruction.

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