TUMOR-PROMOTING PHORBOL ESTERS STIMULATE C3b AND C3b' RECEPTOR-MEDIATED PHAGOCYTOSIS IN CULTURED HUMAN MONOCYTES*

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When the third component of complement, C3, is proteolytically activated, the resulting fragment, C3b, binds covalently to receptive surfaces (1). In the presence of serum factors βH (H)¹ and I, C3b is rapidly cleaved to yield an altered form, C3b’, which is relatively stable in serum (2). Human monocytes recognize both C3b and C3b’, and several lines of evidence indicate that monocytes bear separate receptors for each of these two forms of C3 (3–5). Both C3b and C3b’ receptors efficiently mediate the attachment of erythrocytes coated with their respective ligands, but neither receptor is an efficient mediator of phagocytosis (6).

Resident peritoneal macrophages of the mouse also bind C3-coated erythrocytes without ingesting them (7). However, these cells become capable of ingesting C3-coated erythrocytes after treatment with a soluble factor secreted by T lymphocytes (8–10). These findings prompted us to search for compounds that stimulate the phagocytic capacity of the C3b and C3b’ receptors on human monocytes and to examine the functional relationships between the two C3 receptors.

To obtain suspensions of cultured monocytes for use in these experiments, we adapted the Percoll purification method of Gmelig-Meyling and Waldmann (11) and the Teflon culture method of van der Meer et al. (12). Using these methods, we routinely recover 75–85% of all monocytes present in a blood sample and can observe differentiation of this population without selective cell loss during culture. We find that treatment of these monocytes with tumor-promoting phorbol esters renders their C3b and C3b’ receptors capable of efficiently and independently promoting the phagocytosis of erythrocytes coated with the corresponding ligands and that the capacity of the monocytes to respond to phorbol esters is developmentally regulated.

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Abbreviations used in this paper: AI, attachment index; DNP, rabbit anti-DNP-IgG; DFP, diisopropylfluorophosphatase; DGVB**, 2.5 mM veronal buffer, pH 7.5, 75 mM NaCl, 2.5% dextrose, 0.05% gelatin, 0.15 mM CaCl₂, 0.5 mM MgCl₂; DNP, dinitrobenzene; E, sheep erythrocytes; ElgM, E coated with rabbit anti-E IgG; EC3b, ElgM coated with complement components C1, C4, C2, and C3; EC3b’, EC3b treated with I; EC3d, EC3b’, treated with trypsin; H, β1H; HSA, human serum albumin; I, C3b-inactivator; NHS, normal human serum; NSE, non-specific esterase; PBS, phosphate-buffered saline with Ca and Mg ions; PBSG, PBS containing 3 mM glucose; PD, PBS deficient in Ca and Mg ions; PDD, phorbol-12,13-didecanoate; 4-α-PDD, 4-α-phorbol-12,13-didecanoate; 4-α-PHR, 4-α-phorbol; PI, phagocytic index; PLI, poly-l-lysine; PMA, phorbol-12-myristate-13-acetate; PTA, phorbol-12,13-20-triacetate; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate.

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Materials and Methods

Reagents. Purified human C3 was a gift from Drs. S. K. Law and R. P. Levine, Washington University School of Medicine, St. Louis, MO. Cordis Laboratories Inc. (Miami, FL) supplied C1, C2, C4, I, cobra venom factor, and anti-sheep erythrocyte IgG and IgM. Fab fragments of mouse monoclonal anti-C3 IgG (Bethesda Research Laboratories, Rockville, MD) were made by the method of Porter (13) and were purified by affinity chromatography on C3zymosan (14). Soybean trypsin inhibitor (SBTI), trypsin, and papain were products of Worthington Biochemicals (Freehold, NJ); phorbol-12,13-didecanoate (PDD), 4-α-phorbol-12,15-didecanoate (4-α-PDD), phorbol-12,13,20-triacetate (PTA), and 4-α-phorbol (4-α-PHR) were supplied by Vega-Fox Biochemical Division (Tucson, AZ). Percoll, Sepharose 4B, and Ficoll-Paque were products of Pharmacia Fine Chemicals (Div. Pharmacia Inc., Piscataway, NJ). Sigma Chemical Co. (St. Louis, MO) supplied 3,3'-diaminobenzidine (DAB), α-naphthylbutyrate, paranosaline, methyl green, colchicine, podophyllotoxin, disopropyl fluorophosphate (DFP), poly-L-lysine (PLL), cycloheximide, Triton X-100, phenolphthalein glucuronic acid, β-glucuronidase, phorbol-12-myristate-13-acetate (PMA), and conconavalin A. K76COOH was a generous gift of Dr. K. Inoue, Osaka University Medical School, Osaka, Japan. Normal human serum was obtained using type A, Rh + blood from healthy volunteers as described (15).

Radioiodination and Gel Analysis. C3, rabbit anti-sheep erythrocyte IgG, and monoclonal anti-C3 Fab fragments were iodinated by the iodoam method as described (16), yielding proteins with specific activities of 2 × 10⁵–2 × 10⁶ cpm/μg. No alterations in the hemolytic activity of C3 was measurable after iodination. The polypeptide structure of erythrocyte-bound iodinated C3 was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (17) after base-catalyzed scission of the ester linkage between C3 and the erythrocyte surface (14).

Enzyme and Protein Assays. Monocyte pellets were lysed with 0.5% Triton X-100 and the activity of β-glucuronidase in the lysate was determined by the hydrolysis of phenolphthalein-β-glucuronide as described (18). Units of enzyme activity were calculated by comparison with a standard β-glucuronidase preparation (Sigma Chemical Co.) assayed in parallel. Protein concentration was determined by the method of Lowry et al. (19).

Cytochemistry. 1–2 × 10⁶ cells were deposited on glass slides using a Shandon cytocentrifuge (Shandon Southern Products Ltd., Astmoor, U.K.) set to 500 rpm. After air drying, the slides were stained to reveal nonspecific esterase activity (20), peroxidase activity (21), or nuclear morphology (Diff-Quik; Harleco, Gibbstown, NJ).

Ligand-bearing Erythrocytes. Sheep erythrocytes (E) (Laboratory Animal Research Center, the Rockefeller University) were coated with either rabbit anti-E IgG or IgM according to Nelson et al. (22), yielding E1gG or E1gM, respectively. The IgG was adsorbed with Staphilococcus aureus (Bethesda Research Laboratories) to remove residual IgG and was used at a subagglutinating concentration that corresponded to ~200 IgG/E by hemolytic analysis (23). In some experiments, radiiodinated anti-IgG was used to determine the number of IgG molecules per E. Binding of 125I-IgG to E was a linear function of the quantity of IgG used, and aggregation of E began to occur at concentrations exceeding 100,000 IgG/E. Unless otherwise stated, E1gG were prepared so as to bear ~40,000 IgG/E. E1gG were used up to 2 d after their preparation.

C3-bearing E were prepared using a modification of the method of Law et al. (2). Briefly, 10⁶ E1gG in 1 ml veronal buffer with dextrose and gelatin (DGVB++) were treated sequentially with 40 U C1 (0°C, 40 min), 100 U C4 (30°C, 40 min), and 100 U C2 (30°C, 10 min), with a wash in DGVB++ between steps. The amounts of complement components C1, C4, and C2 were adjusted to yield 200 hemolytic sites per E. The resultant E1gMC142 were immediately washed in DGVB++, resuspended in 0.5 ml DGVB++ containing 75 μg C3, incubated for 30 min at 37°C, and washed twice in DGVB++. The resulting cells (EC3b) were strongly immune adherence positive (2) and were agglutinatable by anti-C3c and anti-C3d (Accurate Chemical & Scientific Corp., Westbury, NY). Furthermore, all cell-bound 125I-C3bα polypeptide exhibited an Mr of 130,000 on SDS polyacrylamide gels.

The procedure described above resulted in ~100,000 C3 molecules deposited per E. To obtain E bearing different amounts of C3, the quantity of C3 added to the E1gMC142 was varied. The amount of C3 attached to the E was quantitated by the binding of 125I-Fab-anti-C3. In some experiments we confirmed our quantitation using 125I-IgG.
Fig. 1. SDS electrophoresis of erythrocyte-bound C3. Erythrocytes were coated with 125I-C3 and prepared for electrophoretic analysis of their bound C3 as described in Materials and Methods. An autoradiogram of the resulting gel is shown. Lane A, 125I-C3; lane B, EC3b; lane C, EC3b'; lane D, EC3d.

A portion of the EC3b preparation was treated with factor I to convert cell-bound C3b to C3b': 0.5 x 10^8 EC3b in 0.5 ml DGVB++ were incubated with 75 U of I, which had been treated with DFP as described (2). After 2 h at 37°C, the conversion of C3b to C3b' was complete as assessed by SDS gel analysis of iodinated C3; all 125I-C3α polypeptide appeared as a 60,000-Mr fragment. However, a small amount of C3b remained on the surface as assessed by a weakly positive immune adherence reaction. Therefore, the factor I treatment was supplemented by a 5-min incubation with 1% human serum that had been exhaustively adsorbed with sheep E and decomplemented with cobra venom factor (20 U/ml, 37°C, 2 h). This treatment abolished remaining immune adherence activity and did not alter the SDS gel pattern of radiiodinated, cell-bound C3b'. EC3b' could be agglutinated by both anti-C3c and anti-C3d serum. EC3b' were treated with trypsin to convert cell-bound C3b' to C3d: 0.5 x 10^8 EC3b' in 0.5 ml DGVB++ were incubated with 5 μg trypsin for 5 min at 37°C. The trypsin was inhibited with a 10-fold excess of SBTI, and the cells were washed three times. The resultant EC3d were immune adherence negative, could not be agglutinated by anti-C3c, could be agglutinated with anti-C3d, and bore a reduced quantity of 125I-C3, which appeared as a 30,000-Mr species on SDS gels. Fig. 1 shows SDS gel analysis of 125I-C3 eluted from EC3b, EC3b', and EC3d prepared as described above. EC3b, EC3b', and EC3d were stored on ice in DGVB++ containing penicillin and streptomycin for up to 10 d with no loss of rosette-forming capacity and <10% loss of 125I-C3.

Ligand-bearing Culture Surfaces. IgG-bearing surfaces were prepared by sequentially treating tissue culture plastic with PLL, dinitrophenol (DNP), and anti-DNP exactly as described by Michl et al. (24). Complement was deposited on IgG-bearing surfaces using purified C1q, C4q, C2q, and C3 as described for the production of EC3b and EC3b'. The resulting surfaces bore both anti-DNP-IgG and either C3b or C3b'.

Phagocytosis and Attachment Assays. The ability of mononuclear phagocytes to bind and ingest ligand-coated E was measured using a microassay performed with Terasaki plates (3040; Falcon Labware, Oxnard, CA). Terasaki plates were pretreated with 5 mg/ml human serum albumin (HSA) for 30 min at room temperature and washed. (Phagocytic indices were depressed if this step was omitted.) Phagocytes were brought to a concentration of 5 x 10^7/ml in phosphate-buffered saline (PBS) containing 3 mM glucose (PBSG), and 5 μl of this suspension was added to each Terasaki well. After 45 min at 37°C, the plates were washed by dipping in PBS, and
the level of fluid in each well was reduced to <2 µl by aspiration. 5 µl of ligand-bearing E (10³/ ml in DGVB++) was added to each well, and the plates were again incubated at 37°C for 45 min. Nonattached E were removed from wells with a controlled stream of buffer applied by passing PBS deficient in Mg and Ca ions (PD) through a 10-µl capillary micropipette (Yankee Micropet; Clay Adams, Parsippany, NJ) using a pressure head of 50 cm of H2O. Some wells were dipped in distilled water five times for 1 s each to lyse uningested E, and then all samples were fixed with 2.5% glutaraldehyde in PD. The number of attached or ingested E was determined by phase-contrast microscopy using an inverted microscope (Carl Zeiss, Inc., New York) with a 40X water-immersion objective. The percent of phagocytes that attached or ingested E multiplied by the average number of E per phagocyte is expressed as attachment or phagocytic index, respectively. At least 100 cells in duplicate wells were counted for each data point.

E-bearing Culture Surfaces. E were attached as a monolayer to Terasaki wells by the following protocol. Surfaces were treated with 0.1 mg/ml PLL in PD for 30 min at room temperature. After a thorough wash in PD, each well received 10⁶ E in PD and plates were centrifuged at 400 g for 5 min. Excess E were removed by vigorous dipping in PD, and remaining PLL was neutralized by incubation with 1 mg/ml HSA for 30 min. The adherent E were converted to ghosts (G) by incubating them five times for 1 s in 5 mM sodium phosphate, pH 8.

Preparation of Percoll Gradients. Preformed Percoll gradients were constructed by a modification of the procedures published by several laboratories (11, 25-27). Percoll was first brought to isotonicity by the addition of 0.1 vol of 10X-concentrated PD. 1 ml normal human serum (NHS), 14.7 ml PBS, and 22 ml isotonic Percoll were mixed in each of four sterile 50-ml polycarbonate centrifuge tubes (Dupont Instruments, Newton, CT), and the tubes were then centrifuged for 25 min at 18,000 rpm at 5°C in a Sorvall SS-34 rotor. The resulting gradients were held on ice for up to 5 h before use.

Isolation of Human Monocytes. “Buffy coat” from 1 U of fresh human blood was prepared by the Greater New York Blood Center as described (28). We obtained the preparation 2-3 h after the blood was drawn. The buffy coat (average volume of 40 ml) was diluted twofold with saline containing 1 mM EDTA, pH 7.4, and mononuclear cells were separated from other formed elements of the blood by centrifugation on step gradients of Ficoll-Paque as described by Boyum (29). The mononuclear cells were washed three times in cold RPMI 1640 (Gibco Laboratories, Grand Island, NY) to remove platelets, and the cells were suspended to 2-5 × 10⁷/ml in RPMI made 12.5% in NHS. 5 ml of this suspension was layered on each Percoll gradient and the tubes were promptly centrifuged at 1,500 g for 25 min at 5°C in a swinging bucket rotor. The resulting bands were easily detected by light scattering. The midpoint between the two major bands was marked and the entire gradient was fractionated into an upper half (band I) and a lower half (band II). Cells from bands I and II from several gradients were pooled and promptly washed twice in cold RPMI 1640.

Culture of Human Monocytes. Purified monocytes were cultured in screw cap Teflon (PFA) jars (Savillex, Minnetonka, MN) or in Teflon (FEP) beakers (Nalge Co., Rochester, NY) covered with foil. In experiments where continuous observation was required, cells were cultured in a Teflon film apparatus described by van der Meer et al. (12). Purified monocytes were added to Teflon vessels at 10⁶/ml in RPMI 1640 and cultures were incubated at 37°C in a 5% CO₂ atmosphere.

Viability and Efficiency of Plating. The plating efficiency of phagocytes on a tissue-culture plastic surface treated with HSA was measured by using a hemocytometer to count the number of cells in culture fluids before and after attachment. The data obtained by this method were confirmed by cell counts made on low-magnification photographs taken before and after washing away nonadherent cells. Cell viability was assessed by trypan blue (Gibco Laboratories) exclusion.

Results

Table I describes a purification of human monocytes that is representative of >40 separate preparations. Mononuclear cells were first isolated by centrifugation on step gradients of Ficoll-Paque. In the
second step, monocytes were separated from lymphocytes by Percoll gradient centrifugation. Band I (88–95% monocytes) was centered on fractions of specific gravity 1.065 and was separated from the lymphocyte band (band II, specific gravity 1.071) by nearly a centimeter, which allowed easy fractionation. Importantly, band I contained 70–85% of all the monocytes in the starting material. Only 2–8% of the monocytes could be recovered in the remainder of the gradient (band II). Thus, the purified monocyte fraction routinely contains >95% of the recoverable monocytes. The cells in band I were identified as monocytes by a number of morphologic, histochemical (nonspecific esterase [NSE] and peroxidase staining), and functional (Fc, C3b, and C3b' receptors) criteria (Tables I and II).

The purified monocytes (band I) were cultured in Teflon vessels to which they adhere lightly or not at all. Cell number remains relatively constant during culture (Table II), which indicates that the monocytes do not die or divide. Rather, as described previously (28, 30, 31), the cells differentiate into macrophages. They lose peroxidase granules and retain nonspecific esterase activity, but increase in size, protein content, and lysosomal enzyme content. Cell fusion begins after 5 d in culture, resulting in a variable number of binucleate or multinucleate cells (Table II).

**Receptor Activity of Cultured Monocytes.** Except after the first 24 h in culture, mono-
cytes showed a very high plating efficiency (Table II). Therefore, the adherent cell population derived from a Teflon culture represents the whole population and not a selected subpopulation. When adherent cells from Teflon culture were tested for their ability to bind IgG-, C3b-, or C3b'-coated erythrocytes, nearly all phagocytes (~90%) showed strong rosetting with all three types of particles. During differentiation, the fraction of phagocytes capable of binding the ligand-coated E remained high, whereas the number of E bound per phagocyte increased, which resulted in a progressively rising attachment index (Fig. 2).

The capacity of monocytes to phagocytose EIgG also increased during differentiation, but the ability of cultured monocytes to ingest EC3b or EC3b' remained remarkably low (Fig. 2): we confirm the results of Newman et al. (6) that, upon extended culture, a small amount of complement-mediated phagocytosis can be seen, that C3b' is a more effective ligand than C3b (Fig. 2), and that the amount of C3-mediated phagocytosis seen varies from preparation to preparation. The failure of monocytes to ingest C3-coated E is not caused by an inhibitory effect of C3b or C3b' on the monocytes phagocytic capacity since rosetted EC3b or EC3b' are readily ingested upon the addition of anti-E IgG (data not shown).

Modulation of Complement Receptor Activity in Cultured Monocytes. Ligand-bearing substrates were constructed using ligand-coated E. C3-coated and control E were attached as a monolayer to PLL-coated plastic, the E were hypotonically lysed, and cultured monocytes were allowed to attach to and spread on the resulting G monolayer. The receptor activity on the monocyte's nonadherent surface was then determined with a rosette assay. Table III shows that monocytes on IgM-coated G monolayers retained full ability to bind EIgG, EC3b, and EC3b'. When plated on IgG-bearing G, the Fc receptors modulated, i.e., EIgG no longer were bound to the cells' nonadherent

![Graph showing development of complement receptor activity and Fc receptor activity on monocytes cultured in Teflon vessels.](image-url)
surfaces, whereas their C3b and C3b' receptor activity remained unaltered. In a similar fashion, when monocytes were plated on C3b-bearing G, only the C3b receptor activity modulated, and when spread on EC3b'-coated G, only the C3b' receptor activity modulated (Table III).

In a second type of experiment, C3 was attached directly to the plastic substrate by constructing complement enzymes on antibody-coated surfaces (Table IV). C3b or C3b' receptor modulation occurred only when the corresponding ligand was deposited on the surface. In contrast to the C3b receptors, the C3b' receptors seldom disappeared completely when the cells were plated on a C3b' substrate: usually a large percentage of macrophages had a number of erythrocytes still adherent (Tables III and IV). These results agree qualitatively with those of Arend and Massoni (32), who used

| Table III |
| Modulation of Receptor Activity on Monocytes Spread on Monolayers of Ligand-bearing Erythrocyte G* |
| Surface | Test particle |
| --- | --- | --- |
| G IgM | 100 | 100 | 100 |
| G IgG | 0 | 130 | 104 |
| G C3b | 99 | 17 | 102 |
| G C3b' | 105 | 103 | 43 |

* E bearing the indicated ligands were attached to PLL-plastic surfaces and lysed as described in Materials and Methods. Freshly isolated or cultured monocytes were allowed to adhere to these surfaces for 45 min at 37°C, then the receptor activity on the apical surface of the phagocytes was assessed with a rosette assay. The results are averaged for seven separate experiments from which the AI obtained on control (G IgM) surfaces was first normalized to 100. Parallel experiments showed that the attachment index obtained for phagocytes on G IgM surfaces was essentially identical to that obtained with phagocytes spread on tissue culture plastic and that the plating efficiency of monocytes on G surfaces was similar to that obtained with plastic surfaces.

| Table IV |
| Modulation of Receptor Activity on Mononuclear Phagocytes Spread on Ligand-bearing Culture Surfaces* |
| Surface | Test particle |
| --- | --- | --- | --- |
| HSA | 750 (87%) | 1450 (100%) | 960 (87%) |
| IgG | 630 (99%) | 1330 (100%) | 55 (19%) |
| IgGC1 | 520 (75%) | 1290 (100%) | 37 (15%) |
| IgGC14 | 620 (73%) | 1450 (100%) | 33 (14%) |
| IgGC142 | 610 (70%) | 1400 (100%) | 26 (13%) |
| IgGC1423b | 77 (20%) | 1400 (100%) | 30 (8%) |
| IgGC1423b' | 599 (82%) | 650 (81%) | 72 (21%) |

* Tissue culture surfaces were coated with DNP as described in Materials and Methods. They were then incubated with HSA or anti-DNP-IgG, followed by the indicated complement components. Day-8 cultured monocytes were allowed to adhere to the indicated surfaces for 45 min at 37°C. Receptor activity on the apical surfaces of the cell was assessed with a rosette assay using the indicated test particles. Results are expressed as attachment indices, and the percent of phagocytes binding at least one E is given in parentheses.
mouse complement as a ligand.

**Effect of PMA on C3b and C3b’ Receptor Function.** Cultured human monocytes can be stimulated to take up complement-coated erythrocytes by treatment with the tumor promoter, phorbol myristate acetate (PMA). A dramatic increase in the phagocytic index for both EC3b and EC3b’ was seen after treatment of phagocytes with as little as 3 ng/ml PMA (Table V). The ability of PMA to stimulate C3-mediated phagocytosis appears to be related to the ability of this drug to promote tumorogenesis, as the nontumorigenic PMA-analogue PT A, 4-α-PDD, and 4-α-PHR were inactive in stimulating complement-mediated phagocytosis. The active tumor-promoting derivative PDD, however, did stimulate phagocytosis (Table V).

The effects of the active phorbol esters (PMA and PDD) on the C3 receptors do not reflect a general enhancement of cellular phagocytic capacities. PMA-treated and control monocytes ingested similar numbers of EIgG. Moreover, PMA did not promote ingestion of E attached to cultured monocytes with concanavalin A (Table V). Thus, the stimulatory effect of PMA on receptor function appears specific for the C3 receptors.

To confirm that the phagocytosis of EC3b and EC3b’ by PMA-stimulated monocytes was not caused by trace contamination of anti-EIgG, all Fc receptor activity was depleted from the nonadherent surface of the phagocytes by plating them on immune complex-coated surfaces. EC3b and EC3b’ were still phagocytosed by PMA-stimulated, mature monocytes depleted of Fc receptors (Table VI).

The ability of monocytes to respond to PMA is developmentally regulated. Freshly explanted monocytes did not phagocytose EC3b or EC3b’ even after PMA stimulation, but with time in culture they gained the capacity to ingest C3-bearing erythrocytes in response to PMA treatment (Fig. 2).

To further characterize the C3b and C3b’ receptors, we studied the effect of ligand density on binding and phagocytosis of EC3b and EC3b’. E were prepared bearing

### Table V

**The Effect of PMA and Its Analogues on the Phagocytosis of Ligand-bearing E by Cultured Monocytes**

| Present during plating | Test particle |  
|-----------------------|---------------|
|                        | EC3b | EC3b’ | EIgG | ECon A‡ |
| 0                     | 14 (8%) | 76 (26%) | 1,414 (100%) | 0 (0%) |
| PMA, 3 ng/ml          | 146 (11%) | 151 (21%) | 1,075 (100%) | 4 (2%) |
| PMA, 10 ng/ml         | 464 (26%) | 388 (38%) | 1,275 (100%) | 27 (12%) |
| PMA, 30 ng/ml         | 454 (56%) | 511 (65%) | 1,070 (100%) | 28 (13%) |
| PMA, 100 ng/ml        | 525 (58%) | 785 (75%) | 1,367 (100%) | 55 (29%) |
| PDD                   | 332 (55%) | 680 (96%) | 580 (83%) | 35 (16%) |
| 4-α-PDD               | 7 (6%) | 65 (21%) | 1,195 (100%) | 0 (0%) |
| PTA                   | 19 (12%) | 53 (17%) | 1,005 (100%) | 0 (0%) |
| 4-α-PHR               | 31 (14%) | 57 (21%) | 1,440 (89%) | 0 (0%) |

* Day 6 cultured monocytes were incubated for 45 min with various concentrations of PMA or with PDD, 4-α-PDD, PTA, or 4-α-PHR at 30 ng/ml. Cells were then washed and the phagocytic index was assessed with the indicated ligand-bearing E. The percent of phagocytes ingesting at least one E is given in parentheses. Under all conditions shown and with all ligand-bearing E used, AI exceeded 1,000.

‡ E coated with concanavalin A.
FC Receptor Function Is Not Required for PMA-stimulated Phagocytosis of EC3b and EC3b*  

| Particle offered | Surface | DNP | DNP-anti-DNP IgG | -  | +PMA | -  | +PMA |
|------------------|---------|-----|-----------------|----|------|----|------|
| EC3b             | DNP     | 8   | 815 (79%)       | 7  | 330  (58%) |
| EC3b'            |         | 190 (36%) | 807 (87%) | 22 (14%) | 482 (74%) |
| ELgG             |         | 860 (80%) | 1390 (94%) | 33 (19%) | 40 (27%) |

* After 7 d in culture, monocytes were plated on DNP-anti-DNP IgG-coated surfaces or on control DNP-coated surfaces in the presence or absence of 30 ng/ml PMA. The cells were then washed and the PI was measured with EC3b, EC3b', or ELgG. The percent of cells ingesting at least one E is given in parentheses.

Fig. 3. Effect of ligand density of the phagocytosis of EC3b, EC3b', or ELgG. After 5 d in culture, monocytes were allowed to adhere to plastic surfaces either in the absence (○) or presence (●) of 30 ng/ml PMA. Cells were then washed and both the AI and the PI were determined using E bearing various amounts of C3b, C3b', or IgG.

different numbers of C3b, C3b', or IgG, and both attachment and phagocytic indices were determined with 5-d-cultured monocytes in the presence or absence of PMA pretreatment (Fig. 3). Approximately 15,000 molecules of C3b/E produced half-maximal rosetting, and even fewer C3b' (5,000/E) yielded half-maximal rosetting. Approximately 10,000 IgG/E were needed for half-maximal rosetting.

In the absence of PMA, a small amount of complement-mediated phagocytosis was seen in cells after a number of days in culture (Fig. 2). Under these conditions, at least 30,000 C3b/E were required to produce detectable ingestion. Treatment of cultured
monocytes with PMA causes a qualitative change in the physiology of the C3b and C3b' receptors: these receptors can now mediate phagocytosis. The density of ligands required for half-maximal phagocytosis by PMA-treated phagocytes is similar for IgG and C3b' (~10,000/E; Fig. 3), which suggests that in appropriately stimulated cells, the receptors for these two ligands are similar in their ability to generate a phagocytic signal. PMA-stimulated cells do ingest C3b-coated E, but a 10-fold-higher density of C3b is required to match the phagocytosis mediated by 10,000 molecules/E of either C3b' or IgG.

PMA has been shown to cause release of proteases from macrophages (33), and the C3b receptor has been shown to facilitate the cleavage of cell-bound C3b to C3b' (34). It was possible, therefore, that enzyme(s) secreted by PMA-stimulated monocytes cleaved E-bound C3b to C3b' and that the resultant C3b' mediated phagocytosis through interaction with the C3b' receptor. To test this possibility, monocytes were plated on C3b'-coated surfaces to modulate their C3b' receptors. The monocytes were treated with PMA to stimulate the activity of their C3 receptors, and were then incubated with EC3b. Despite a significant reduction in C3b' receptor function, these monocytes ingested as many EC3b as monocytes plated on control (IgM) surfaces (Table VII). Conversely, monocytes plated on C3b-coated substrates phagocytosed virtually no C3b-coated E, even though their C3b' receptors remained functional (Table VII). In other experiments, K76COOH, a potent inhibitor of I (35), at a concentration of 1 mg/ml had no inhibitory effect on the uptake of EC3b by PMA-stimulated monocytes (data not shown). Finally, we have found that both Ca and Mg ions (0.15 and 0.25 mM, respectively) are required to support optimal binding and phagocytosis of EC3b' by mononuclear phagocytes, but are not required for optimal binding and phagocytosis of EC3b (Table VIII).

These results indicate that after stimulation by PMA, the C3b and the C3b' receptors function independently of one another in promoting the ingestion of E coated with the corresponding ligands. Although they do not exclude the possibility that monocyte-derived proteases cleave some of the ligated C3b molecules, they provide direct evidence that EC3b are ingested via the monocyte's C3b receptors.

The stimulatory effect of PMA on C3 receptor-mediated phagocytosis is relatively stable and occurs rapidly. The effect was observed when the PMA was added for 45 min and washed away before addition of the ligand-coated E (Table V), or when

### Table VII

| Surface | Test particle | EC3b | EC3b' |
|---------|---------------|------|-------|
| GlG     | 660 (80%)     | 292  (70%) | 391 (76%) |
| GC3b    | 945 (71%)     | 13 (6%)     | 244 (60%) |
| GC3b'   | 775 (84%)     | 300 (52%)   | 160 (53%) |
| GlG     | 5 (2%)        | 331 (75%)   | 450 (73%) |

* E bearing the indicated ligands were attached to PLL-plastic surfaces and lysed as described in Materials and Methods. Day-6 cultured monocytes were allowed to adhere for 45 min at 37°C in the presence of 75 ng/ml PMA. The cells were then washed and the PI was measured with EC3b, EC3b', or ElG. The percent of cells ingesting at least one E is given in parentheses.
TABLE VIII
Effect of Divalent Cations on the Binding and Phagocytosis of Ligand-coated E

| Divalent cations present | Test particle | | | | |
|-------------------------|--------------|---|---|---|---|
|                         | EC3b         | EC3b' | ElgG |
|                         | AI | PI | AI | PI | AI | PI |
| 0.5 mM Ca++ and 0.5 mM Mg++ | 2,292 | 216 | 2,213 | 260 | 2,210 | 528 |
| 0.5 mM Ca++            | 2,222 | 181 | 367 | 101 | 2,028 | 374 |
| 0.5 mM Mg++           | 2,333 | 159 | 471 | 98 | 2,264 | 375 |
| None                   | 2,090 | 130 | 177 | 11 | 2,400 | 336 |

*7-d cultured monocytes were incubated for 45 min in PBSG containing 30 ng/ml PMA. The cells were then thoroughly washed in phosphate buffer free of divalent cations and the AI and PI were determined using the indicated test particles suspended in buffer containing the indicated cations.

TABLE IX
Effect of Drugs on PMA-stimulated Phagocytosis of Ligand-bearing E

| Present during plating | Present during phagocytosis | Test particle | | | |
|------------------------|-------------------------------|--------------|---|---|---|
|                        |                               | EC3b         | EC3b' | ElgG |
|                        |                               | AI | PI | AI | PI | AI | PI |
| —                      |                               | 22 (11%) | 84 (27%) | 995 (90%) |
| PMA                    |                               | 414 (47%) | 654 (86%) | 1060 (89%) |
| —                      | PMA                           | 415 (65%) | 488 (70%) | 1070 (95%) |
| PMA                    | Colchicine                    | 61 (21%) | 142 (27%) | 570 (88%) |
| PMA                    | Podophyllotoxin               | 87 (25%) | 134 (22%) | 590 (85%) |
| Cycloheximide and PMA  | Cycloheximide                 | 430 (43%) | 616 (88%) | 891 (84%) |

*After 6 d in culture, monocytes were allowed to adhere to plastic surfaces for 45 min at 37°C in the presence or absence of 30 ng/ml PMA as indicated. When used, PMA (30 ng/ml) and cycloheximide (both at 10^{-5} M) were incubated with the cells for 10 min before the addition of PMA. Cells were then washed and incubated with ligand-coated E in the presence or absence of colchicine, podophyllotoxin (both at 10^{-5} M), or PMA (30 ng/ml). Results are expressed as PI, and the percent of cells ingesting at least one E is given in parentheses.

added at the same time as the E (Table IX). When PMA was added to phagocyte-
EC3b or EC3b' rosettes, interiorization of bound E occurred rapidly (within 10 min, data not shown).

Griffin and Griffin (9) and Griffin and Mullinax (10) have shown that T cell factor-
mediated activation of complement receptors in mouse macrophages is not affected by
cycloheximide but is blocked by treatment of the macrophages with agents that
depolymerize microtubules. We have found similar effects with the PMA-stimulated phagocytosis of EC3b and EC3b': when added to washed, PMA-stimulated cells, the microtubule depolymerizing agents colchicine and podophyllotoxin reversed the effect of PMA; complement receptor-mediated phagocytosis was no longer seen (Table IX). Cycloheximide, on the other hand, caused no alteration in the PMA-stimulated phagocytosis of EC3b and EC3b'.
Discussion

Our results confirm that monocytes bear receptors for C3b and C3b' but not for C3d (3-6), and that the C3b and C3b' receptors function independently of one another in a single cell (Tables III, IV, VII, and VIII) (3). The presence of separate receptors for two closely related ligands on a single cell type may be puzzling, but it is not without precedent. Mouse (36) and human (37) macrophages bear several distinct receptors for the Fc regions of IgG. It is possible that multiple C3 receptors are required because different forms of C3 may predominate on different particles in vivo. Per molecule of particle-bound ligand, the C3b' receptor is 3 times more efficient in promoting attachment and 10 times more efficient in promoting ingestion than the C3b receptor (Fig. 3). Further, C3b' is relatively stable in serum, whereas C3b can often be rapidly degraded (2). Thus, in vivo, the outcome of an interaction between a mononuclear phagocyte and a C3-coated particle is likely to be governed by the cell's C3b' rather than its C3b receptors.

Activation of C3 and C3b' Receptors by Phorbol Esters. C3 is widely recognized as an essential opsonic ligand (38). Thus, it is puzzling that the C3 receptors on unstimulated human (6), mouse (7), and guinea pig (39) mononuclear phagocytes do not mediate the ingestion of E coated with the corresponding ligand. Mouse and guinea pig C3 receptors can be activated by lymphokines and inflammatory stimuli (8, 39, 40). It seems likely, therefore, that PMA is mimicking a similar regulatory substance that is the physiological activator of these receptors in man.

Several lines of evidence indicate that cells bear specific membrane receptors for phorbol esters (41, 42). Thus, the failure of PMA to activate C3 receptors on freshly explanted monocytes could reflect a lack of receptors for PMA on these cells. We think this unlikely because PMA stimulates adherence, spreading, and H2O2 secretion by freshly explanted monocytes (28). Moreover, compounds such as interferon that inhibit the maturation of monocytes also block the acquisition of PMA-stimulatable C3 receptor activity (S. D. Wright and S. C. Silverstein, unpublished observations). Once human monocytes reach an appropriate stage of differentiation, PMA induces activation of C3 receptors within a few minutes and without a requirement for protein synthesis (Table IX). For these reasons, we think that qualitative changes that occur as monocytes mature to macrophages govern the responsiveness of C3 receptors to PMA, and that PMA inducibility of C3 receptor function may be a marker for this transition.

We do not understand the mechanism(s) by which PMA makes complement receptors capable of generating a phagocytic signal. Possible mechanisms include: (a) insertion of competent receptors into the plasma membrane from an internal pool; (b) covalent modification (e.g., phosphorylation, methylation, etc.) of existing receptors; and (c) insertion of coupling factors that might interact with ligated C3 receptors to generate a phagocytic signal. With respect to this last possibility, it should be noted that Fc receptors are constitutively competent to mediate phagocytosis. Thus, if other factors are required to promote complement receptor-mediated phagocytosis, the putative factors that interact with C3 receptors must be distinct from those that interact with Fc receptors.

Comparison of Murine and Human C3 Receptors. This study confirms an interesting difference in the behavior of C3 receptors of mouse and human mononuclear phagocytes. Complement receptors of unstimulated mouse peritoneal macrophages
appear to be immobile because they are not modulated when those cells are plated on C3-bearing surfaces (24). Those macrophages do not ingest C3-coated E. Complement receptors of lymphokine-treated (10) or thioglycollate broth-elicited macrophages (24) appear to be mobile because they are modulated by C3-bearing surfaces. These macrophages ingest C3-coated E via their complement receptors. The above findings led Michl et al. (24) to suggest that C3 receptor mobility is required for C3 receptor-mediated uptake of C3-coated particles. The present study and that of Arend and Massoni (32) show that C3 receptors on human monocytes are mobile, at least as measured by their capacity to modulate in response to C3-coated surfaces. Nevertheless, in the absence of stimulation by PMA, these receptors do not mediate phagocytosis of C3-coated E. Thus, receptor mobility may be necessary for C3 receptor-mediated phagocytosis to occur, but the presence of mobile receptors is not sufficient. Evidently, several steps are required to enable C3 receptors to generate a phagocytic signal. We suggest that unstimulated mouse and human mononuclear phagocytes illustrate different intermediate stages in this process.

Summary

Monocytes were isolated in high yield (~80%) and purity (>90%) by Percoll gradient centrifugation and incubated in Teflon culture vessels. Using this culture method, we routinely recovered 80% of the cells originally placed into culture. Studies of the C3b and C3b' receptors of these monocytes showed that the function of both receptors could be dramatically altered by treating the cells with tumor-promoting phorbol esters. Both C3b and C3b' receptors of human monocytes efficiently mediate attachment of erythrocytes coated with the corresponding ligands, but do not promote their ingestion. However, monocytes treated with phorbol myristate acetate (PMA) or phorbol didecanoate ingest C3b- and C3b'-coated erythrocytes. Phorbol esters that are inactive as tumor promoters do not stimulate C3 receptor-mediated phagocytosis. The ability of monocytes to respond to PMA by activation of C3 receptors is developmentally regulated. Freshly isolated monocytes do not take up C3b- or C3b'-coated erythrocytes in response to PMA, but after 3 d of culture they show strong PMA-stimulated uptake. The stimulatory effect of PMA on monocyte C3b and C3b' receptor function occurs within minutes, is stable for hours, is cycloheximide insensitive, and can be inhibited with colchicine. Several lines of evidence indicates that phagocytosis of C3b or C3b'-coated erythrocytes is specifically mediated by the monocytes' C3b and C3b' receptors. First, erythrocytes attached to monocytes with concanavalin A are not ingested when the monocytes are treated with PMA. Second, monocytes plated on IgG-bearing substrates lose Fc receptor activity on their nonadherent surfaces but retain the capacity to ingest C3b- or C3b'-coated erythrocytes after PMA treatment. Third, PMA-treated monocytes plated on C3b-coated surfaces lose C3b receptor activity on their nonadherent surfaces but retain the capacity to ingest C3b'-coated erythrocytes. Conversely, PMA-treated monocytes plated on C3b'-coated surfaces show reduced C3b' receptors activity on their nonadherent surfaces but retain the capacity to ingest C3b-coated erythrocytes.

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