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The complement fragment C3d facilitates phagocytosis by monocytes

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SUMMARY

Two receptors for fragments of C3 are described for human monocytes: CR1 and CR3, which bind C3b and iC3b, respectively. Recently a leucocyte receptor that binds C3dg has also been described, designated CR4. We previously reported that IgM-sensitized sheep erythrocytes that are heavily coated with C3d (EAC3d) can bind to human monocytes that have been cultured in fetal calf serum (FCS). Here we determine whether such binding of C3d-coated targets can lead to phagocytosis, and identify the specific monocyte receptor involved in C3d binding. We confirm that EAC3d bearing greater than 10,000 C3d/cell bind to FCS-cultured monocytes. Furthermore, using non-cultured monocytes, we demonstrate that C3d enhances rosette formation of IgG-coated E and, like C3b and iC3b, C3d augments IgG Fc receptor-mediated phagocytosis. Less than 100 C3d/cell are capable of enhancing phagocytosis, whereas 10,000 or more C3d/cell are required for rosette formation with cultured cells. These results indicate that the C3d-binding receptor is present on peripheral blood monocytes but has poor affinity for target particles coated only with C3d. Anti-CR2 monoclonal antibodies, which recognize the C3d receptor of lymphocytes, do not block EAC3d rosette formation with monocytes. In contrast anti-Mol, a monoclonal antibody against CR3, inhibits EAC3d rosettes by approximately 42%. Anti-CR1 increases this effect, but complete inhibition is not achieved. Ethylenediamine tetraacetate also markedly reduces EAC3d rosetting, reducing the numbers to less than 5%. Thus, the C3d-binding receptor on monocytes, unlike CR4, is metal dependent. Together these data indicate that CR3 is predominantly responsible for C3d binding to monocytes.

INTRODUCTION

The complement component C3 plays an important role in the process of opsonization, and its chemistry and function have been studied in detail. The major cleavage fragment of C3, C3b, is covalently bound to the surface of pathogens during the activation of both the classical (C4b2a) and alternative (C3bBb) complement pathways. On many types of cellular surfaces, the bound C3b is not stable and is cleaved by the plasma enzyme, Factor I (I), to a haemolytically inactive form, iC3b. This fragment, which remains attached to the activator, in the presence of a co-factor is highly susceptible to further cleavage by I, resulting in the retention of a surface-bound 41,000 MW fragment of C3, C3dg, and the release into the fluid phase of a larger fragment of C3 containing all of the β chain and much of the α chain, C3c. Cleavage of C3dg by trypsin, elastase or plasmin generates a 31,000 MW surface-bound fragment, referred to as C3d (reviewed by Berger, Gaither & Frank, 1981–82).

Leucocytes express membrane receptors which recognize these various forms of the C3 molecule. The interaction of leucocyte complement receptors (CR) with C3 fragments plays a key role in several host defence mechanisms, particularly the ingestion of opsonized pathogens by phagocytic cells. Thus far, three distinct C3 receptors have been characterized: CR1, CR2, and CR3. Both CR1 (which binds C3b and iC3b) and CR3 (which binds iC3b) have been shown to enhance IgG Fc receptor (FcR)-mediated ingestion of C3-IgG opsonized particles by neutrophils and monocytes. Under certain conditions CR1 and CR3 may mediate phagocytosis independently of the IgG FcR. CR2, which binds C3d, iC3b and C3dg, is found predominantly on lymphoid cells and presumably does not play a role in phagocytosis (Berger *et al.*, 1981–82; Schreiber, 1984; Ross &

Abbreviations: C3, third component; CR, complement receptor; DVBS, isotonic dextrose Veronal-buffered saline containing Mg^{2+} and Ca^{2+}; E, sheep erythrocytes; EA, IgM-coated E; EAC14, EA bearing C1 and C4; $EAC14_T$, trypsin-treated EAC14; EAC3b, EA bearing the major cleavage fragment of C3, C3b; EAC3bi, EA bearing the I-inactivated form of C3b, iC3b; EAC3d, EA bearing the trypsin-generated fragment of C3, C3d; EDTA, ethylenediamine tetraacetate; EDTA-DVBS, isotonic dextrose without metals (0·0375 μ) containing 0·01 M EDTA and 0·1% gelatin; EDTA-VBS, isotonic VBS without metals containing 0·01 M EDTA and gelatin; FCS, fetal calf serum; H, Factor H; HBSS$^-$, Hanks' balanced salt solution with Ca^{2+} or Mg^{2+}; I, Factor I; PI, phagocytic index, number of E ingested/100 monocytes; VBS + BSA, Veronal-buffered saline containing Ca^{2+} and Mg^{2+} and 2 mg/ml bovine serum albumin.

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Medof, 1985). Several years ago we reported that erythrocytes coated with large numbers of C3d molecules bind to human monocytes cultured in fetal calf serum (FCS) (Inada *et al.*, 1983). Further investigation of this phenomenon by others suggested that phagocytes have an additional receptor, that some have termed CR4, which binds the C3dg fragment of C3 (Weis, Tedder & Fearon, 1984; Vik & Fearon, 1985; Frade *et al.*, 1985). It was thought that binding of C3d-coated erythrocytes to monocytes might reflect the action of this putative new receptor. Here we have attempted to define specific receptor interactions involved in C3d binding to cultured monocytes. These studies also investigate the role of C3d in Fc-mediated phagocytosis in order to determine if C3d, like C3b and iC3b, enhances the ingestion of IgG-coated particles.

MATERIALS AND METHODS

Buffers and media

Isotonic buffers were prepared as described elsewhere: DVBS and EDTA-VBS (Mayer, 1961; Rapp & Borsos, 1970) and EDTA-DVBS (Gaither, Hammer & Frank, 1979). Hypotonic-lysing buffer containing ammonium chloride, potassium bicarbonate and EDTA was obtained from the NIH Media Section. Hanks' balanced salt solution (HBSS), RPMI-1640 medium with L-glutamine and fetal calf serum (FCS), were purchased from Gibco, Grand Island, NY.

Reagents

Reagents were purchased from the following sources: lymphocyte separation medium (LSM), Litton Bionetics, Kensington, MD; percoll, Pharmacia Fine Chemicals, Uppsala, Sweden; bovine pancreatic trypsin (type III, 2 × recrystallized) and soybean trypsin inhibitor (Type I-S), Sigma Chemical Co., St Louis, MO. Partially purified complement components, including human C2, guinea-pig C1, and human C3 inactivator (I) were purchased from Cordis Corp., Miami, FL.

Highly purified IgM and IgG fractions of rabbit anti-Forssman antibody were obtained as described elsewhere (Frank & Gaither, 1970). IgG was radiolabelled (specific activity 0·55–2·2 μCi/μg) with ^{125}I using iodobeads following procedures recommended by the manufacturer (Pierce Chemical Co., Rockford, IL). All purified complement components were of human origin. Factor H (H) and Factor I (I) were purified using an anti-Facot H immunoadsorbent (Gaither *et al.*, 1979). Procedures for purifying human C3 and C4 (Hammer *et al.*, 1981) and bovine conglutinin (Brown *et al.*, 1982) are described elsewhere. C3 was radiolabelled with ^{125}I to a specific activity of 0·045 μCi/μg, using the iodobead method.

Monoclonal antibodies

Monoclonal antibodies to human C3 receptors were obtained from the following sources: an IgG fraction of anti-CR1 (3D-9) (O'Shea *et al.*, 1985) and purified anti-Mol (mouse IgM), Coulter Immunology, HiaLeah, FL; anti-B2 mouse ascites, a gift from Dr L. Nadler; anti-B2 IgM, Coulter Immunology; and OKB7 IgG, Ortho Diagnostics, Rariton, New Jersey. The specificity of anti-Mol for CR3 has been previously documented (Todd *et al.*, 1984) as has that of anti-B2 and OKB7 for CR2 (Iida, Nadler & Nussenzweig, 1983; Rao *et al.*, 1985). Purified mouse IgG and IgM were used as immunoglobulin subclass controls (Coulter Immunology). F(ab′)$_2$ fraction of goat anti-mouse IgM was purchased from Cappel, Cochranville, PA.

Monoclonal antibodies directed against C3 fragments were purchased from Boehringer, Mannheim Biochemicals, Indianapolis, IN. Anti-C3c, anti-C3d and anti-iC3b neo-antigen were radiolabelled with ^{125}I by the iodobead method to specific activities of 0·24, 0·96 and 0·51 μCi/μg, respectively. The radiolabelled antibodies were pre-adsorbed twice at 0° for 20 min with packed sheep erythrocytes. All monoclonal antibody preparations were centrifuged at 80,000 *g* for 30 min before use in binding or inhibition experiments.

Cellular intermediates

Freshly drawn sheep erythrocytes (E) were sensitized with IgM anti-Forssman antibody to form EA. EAC14, and EAC43b and EAC4 ^{125}I C3b were prepared as previously described (Gaither *et al.*, 1983). EAC3b were converted to EAC3bi and EAC3d by either one of two methods. In the first method, EAC3b suspended in EDTA-DVBS was incubated for 90 min at 37° with 6·7 μg/ml H and a 1:200 dilution of I (Gaither *et al.*, 1983). A portion of EAC3bi cells was converted to EAC3d by incubation with trypsin (1 μg/ml) for 45 min at 37°, followed by neutralization with 5 μg/ml soybean trypsin inhibitor, and two washes in DVBS. In the second method, EAC3b were converted to EAC3bi by adding H (100 μg/ml) and I (1:25) to cells suspended in normal ionic strength buffer (VBS + BSA). These conditions do not foster the conversion of EAC3bi to EAC3dg (Ross *et al.*, 1982). After 45 min at 30° the cells were washed twice and resuspended in DVBS. EAC3bi were converted to EAC3d by incubation with 10 μg/ml of trypsin for 30 min at 37° followed by neutralization with 20 μg/l of soybean trypsin inhibitor. As a control, EAC14 were similarly treated with trypsin and neutralized (EAC14$_T$). We observed a greater retention of C3 fragments on cells prepared by this method and adopted it for most of the studies reported here.

Quantification of bound C3 fragments was done by measuring the binding of ^{125}I-monoclonal anti-C3c, anti-iC3b and anti-C3d to the various cellular intermediates. Saturating inputs of the radiolabelled antibodies were added to 100 μl containing $1·5 \times 10^7$ cells and mixed well. After 90 min at 0°, 75 μl from each cell suspension was centrifuged through dibutyl phthalate (Eastman, Rochester, NY) in 400 μl Beckman microfuge tubes (Beckman, High Wycombe, Bucks, U.K.). The cell pellets were retrieved and counted. As controls, binding of the antibodies to EAC14 and to trypsinized EAC14 (EAC14$_T$) was measured.

For IgG sensitization, aliquots of each cellular intermediate were incubated with various concentrations of unlabelled or ^{125}I-IgG anti-Forssman for 15 min at 37° followed by three washes in DVBS. The uptake of ^{125}I-IgG was determined using a Packard Prias gamma counter.

All cellular intermediates were suspended in DVBS for rosetting and phagocytosis studies.

Preparation of monocytes

Human monocytes were isolated by two-step density gradients of Ficoll–Hypaque (LSM) and Percoll (Gmeleg-Meyling & Waldman, 1980) or by elutriation using a 1221 centrifuge (Beckman) equipped with a JE6 elutriator rotor with two Sanderson separation chambers, following a procedure modified from Lionetti, Hunt & Valeri (1980). The purity of both

Table 1. Characterization of C3 fragments that enhance phagocytosis

Cellular intermediate	C.p.m. anti-C3c	C.p.m. anti-iC3b	C.p.m. anti-C3d	Agglutination*	Rosettes (%)	
Experiment 1						
$EAC3b_{200}$	2561	254	6688	4+	45	
$EAC3bi_{200}$	1536	7325	6700	4+	62	
$EAC3d_{1000}$	108	233	27,348	0	40	
EAC14	178	416	1125	0	0	
					No IgG	PI ~600 IgG/cell
Experiment 2						
$EAC3b_{200}$	2571	ND	ND	4+	0	59
$EAC3bi_{200}$	ND	7013	ND	4+	0	100
$EAC3d_{1000}$	511	770	12,370	0	1	120
EAC14	661	879	1778	0	0	29

Binding of ^{125}I-anti C3 fragment monoclonals is described in the Materials and Methods. In each of two experiments, binding of anti-C3c and anti-iC3b to EAC14 was greater than to EAC3d.

* Agglutination of cells with polyclonal anti-C3c was determined macroscopically and microscopically after incubation for 30 min at 37°. Rosette formation was examined using monolayers that had incubated at 37° for 2·5 hr with RPMI + 10% FCS, and phagocytosis with monocytes that were adhered for 30 min at 37° in RPMI. $EAC14_T$ bound similar amounts of anti-C3c, anti-iC3b and anti-C3d as did EAC14 and were ingested similar to EAC14-IgG (PI = 1 without IgG and 29 with IgG).

types of monocyte preparations was >90% by non-specific esterase staining (Technicon), and by morphology. The cells were maintained in an ice bath until use.

Rosette formation

Assays were performed with monocytes in suspension and with cultured monocytes. In the suspended system, 1×10^5 monocytes (in HBSS$^=$) were added to $7{\cdot}5 \times 10^6$ red cells in 12 × 75 mm polypropylene tubes, gently mixed, and centrifuged for 5 min at 50 ***g***. The pelleted cells were slowly rotated at 37° for 30 min in the presence of 5% CO_2. Rosettes were examined microscopically by counting 100 monocytes per sample and determining the percentage with three or more E attached. In assays using cultured cells, $1–1{\cdot}25 \times 10^5$ monocytes in RPMI-1640 plus 10% FCS were added to each of eight chambers of a slide-mounted tissue culture plate (Lab Tek, Naperville, IL) and incubated for 2–5 hr in the presence of 5% CO_2. The plates were gently rinsed three times with RPMI, after which 200 μl RPMI plus 200 μl of the cellular intermediate were added. The chambers were centrifuged at 50 ***g*** for 5 min, and incubated at 37° for 30 min in 5% CO_2. After incubation the chambers were gently rinsed once with RPMI to remove non-adhered red cells. The remaining cells were then fixed with 1·25% glutaraldehyde for 10 min at room temperature and stained with Giemsa.

To examine rosette inhibition, 200 μl of monoclonal antibody or buffer were added to each chamber after the initial incubation and washes. After 30 min at 37° with the antibodies, $F(ab')_2$ fragment of anti-mouse IgM (50 μg/ml) was added to the chambers which received anti-B2 antibody and to respective buffer controls. The cells were incubated further with the 'second' antibody for 30 min at 37°. The sheep cell intermediates were then added and the assay for rosette formation was performed as described earlier.

Phagocytosis

Phagocytosis was examined using monocytes that had been adhered at 37° for 30 min in RPMI without added FCS. The same procedures were followed as with rosette formation except that non-ingested E were removed in two hypotonic lysis steps with ice-cold lysing buffer before fixing and staining the adherent cells. Phagocytosis was quantified by determining the percentage of 100 monocytes ingesting one or more target cells, and the total number of ingested E/100 monocytes (PI) was recorded. Inhibition of phagocytosis by anti-Mol was examined after preincubating adherent monocytes with the antibody or with control mouse IgM for 30 min at 37°. E were then added and phagocytosis was studied as described above.

RESULTS

Characterization of cellular intermediates

When suspended in low ionic strength buffer (DVBS) all EAC3b and EAC3bi preparations were positive in the immune adherence reaction with human E, whereas EAC3d failed in all cases to adhere to human E. Of the three types of cells, only EAC3bi were positive in the conglutinin binding assay (Inada *et al.*, 1983). Both EAC3b and EAC3bi were strongly agglutinated by anti-C3c antiserum, whereas EAC3d were not agglutinated with anti-C3c, as shown by macroscopic or microscopic examination. EAC3b and EAC3bi cells formed prominent rosettes with non-cultured monocytes in suspension, in contrast to EAC3d which failed to bind to the monocytes.

Two representative experiments in which the cell-bound C3 fragments were quantified with monoclonal anti-C3 fragments are shown in Table 1. EAC3d bound less of the ^{125}I-anti-C3c and ^{125}I-anti-iC3b antibodies than did the ESAC14 control. In both

experiments, EAC14$_T$ were similar to EAC14 in the uptake of each of the monoclonal antibodies (not shown). In contrast to EAC3d binding, anti-C3c bound to both EAC3b and EAC3bi; and anti-iC3b, which recognizes iC3b neo-antigen, was taken up only by EAC3bi. We found that low levels of C3b and iC3b deposited by 1·0 U/ml input of C3 could be detected by anti-C3c and anti-iC3b, respectively (not shown). This represents ~80 C3b/cell and ~50 iC3b/cell. Anti-C3d bound equally as well to EAC3b and EAC3bi (Table 1).

The synergistic action of C3d and IgG

Non-cultured peripheral blood monocytes formed prominent rosettes with EAC3b and EAC3bi and failed to bind to EAC3d when examined in a suspended cell system. As we previously reported, EAC3d formed rosettes only with monocytes that had been cultured in monolayers in medium containing FCS Table 1, Exp. 1. However, we noted that EAC3d sensitized with a limited input of IgG (as few as 160 molecules/cell) formed rosettes with peripheral blood non-cultured monocytes in a dose-response fashion (not shown). This observation indicated that C3d, like C3b and iC3b, functions in synergy with IgG to enhance particle binding, and hence may also play a role in phagocytosis.

Table 2. Phagocytosis by monocytes in monolayer (dose–response effect of C3b, iC3b and C3d)

C3 input	EAC3b C3b/cell	PI	EAC3bi iC3b/cell	PI	EAC3d PI
1·0	65	4	33	11	24
10	627	18	366	30	18
50	3041	53	1606	83	28
200	9946	60	5164	85	70
1000	39,847	108	26,059	106	134
1000 w/o IgG		2		10	1
EAC14+IgG		0			
EAC14$_T$+IgG		3			

EAC3b were prepared with varying inputs of ^{125}I-C3 and a portion of each preparation was converted to EAC3bi and EAC3d. The calculation of bound C3b and iC3b was based on ^{125}I-C3 and ^{125}I-iC3b bound to cells after three washes. Since most ^{125}I was associated with C3c and was released from the cells upon trypsinization, this determination could not be made for EAC3d. Cells were coated with ~300 IgG/cell. Phagocytosis was examined as described in the Materials and Methods.

We next examined phagocytosis of intermediates coated with C3 fragments and IgG, using monocytes that were adhered in Lab Tek chambers in the absence of FCS. In the experiment shown in Table 1 (Exp. 2), IgG was added at a concentration that triggered minimal ingestion of EAC14 (~600 IgG/cell). The ingestion of EAC3d-IgG greatly exceeded that of EAC14-IgG and was greater than EAC3b-IgG as well. Ingestion of EAC14$_T$-IgG was similar to EAC14-IgG. In the absence of IgG, none of the cellular intermediates were ingested.

In Table 2, phagocytosis of targets coated with different amounts of the C3 fragments plus a limited quantity of IgG (~300/cell) is shown. In the absence of IgG there was essentially no ingestion of EAC3b or EAC3d and minimal ingestion of EAC3bi (PI = 10). An input of 10 U/ml of C3 to prepare the cellular intermediates was sufficient to augment phagocytosis of each of the three cell types above the controls. Ingestion of EAC3d-IgG was greater than EAC14$_T$-IgG at all inputs of C3 and increased markedly at 200 U/ml of C3 to a level similar to that of the C3b- and iC3b-coated targets. Each of the EAC3d preparations was negative in polyclonal anti-C3c agglutination tests and each failed to bind ^{125}I-anti-C3c and ^{125}I-anti-i3b (not shown).

The concentration of IgG required to trigger phagocytosis of cells coated with each of the C3 fragments was compared (Fig. 1). Without IgG, EAC3b and EAC3d were minimally ingested but, as in the previous experiment, basline ingestion of EAC3bi was somewhat higher without IgG (PI = 20). At a concentration of 120 IgG/cell, C3b, iC3b and C3d augmented phagocytosis above the baseline values. Substantial activity (PI > 100) was achieved with 700 IgG/cell with each of the C3 fragments, whereas EAC14 and EAC14$_T$ showed minimal activity with the same dose of IgG (PI < 20). A concentration of 3500 IgG/cell was required to see substantial ingestion of EAC14 and EAC14$_T$-IgG. Ingestion of EAC3d-IgG was similar to that of C3b- and iC3b-coated cells at each input of IgG.

Interaction of anti-CR antibodies with cultured monocytes

Binding of anti-CR2 monoclonal antibodies, OKB7 and anti-B2, to cultured monocytes was assessed by rosette inhibition and immunofluorescence (not shown). OKB7 had no inhibitory effect on EAC3d rosette formation with cultured monocytes and both purified anti-B2 IgM and unfractionated anti-B2 mouse ascites failed to inhibit EAC3d rosetting in the presence of a second antibody, F(ab′)$_2$ anti-mouse IgM. [In studies of lymphocyte rosette formation, anti-B2 has been noted to require the addition of a second antibody to effectively block rosettes (Iida *et al.*, 1983).] In control experiments the two anti-CR2 monoclonal antibodies blocked EAC3d-rosetting with cells from a cultured B-cell line of cord blood lymphocytes (Gaither *et al.*, 1983). Immunofluorescence staining also showed no binding of anti-B2 monoclonal antibody to cultured monocytes in contrast to prominent staining of cultured B cells. OKB7 was not tested for immunofluorescence.

Table 3 shows that EAC3b rosetting was nearly completely blocked by 3D-9, which had minimal effect on EAC3bi and EAC3d rosetting. In contrast, anti-Mol caused partial but consistent inhibition of EAC3d rosetting (42·1 ± 12·2%), and EAC3bi rosetting (59·2 ± 9·4%). Inhibition of rosetting by the mouse IgM and IgG control antibodies was minimal. When monocytes were incubated with both 3D9 and anti-Mol, inhibition of EAC3bi rosettes was not significantly greater than with anti-Mol alone, whereas EAC3d rosetting was blocked significantly more than with anti-Mol alone ($P < 0·01$ by Student's t-test). However, complete inhibition of EAC3d binding was not achieved. At higher concentrations of anti-Mol antibody (80–100 μg/ml) inhibition of EAC3d binding was similar to that observed with 40 μg/ml antibody (data not shown).

Effect of anti-CR3 antibody on phagocytosis

The effect of anti-Mol on C3d-IgG phagocytic function was

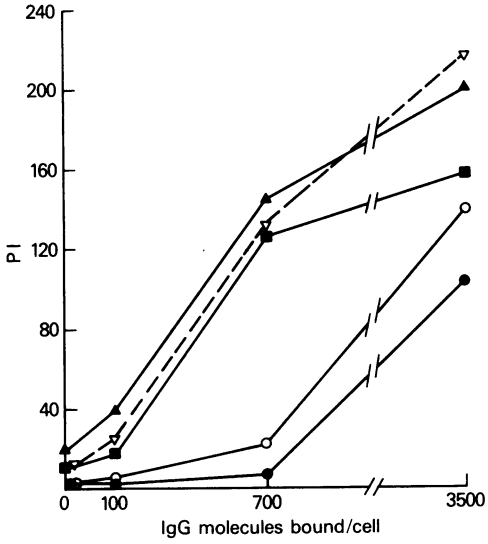

Figure 1. The effect of IgG concentration on ingestion. Target cells included: EAC14 (●), EAC14$_T$ (○), EAC3b (■), and EAC3bi (▲), each prepared with 200 U/ml input of C3 and EAC3d (▽) prepared with 1000 U/ml input of C3. Cells were coated with different concentrations of IgG as shown. Phagocytosis of these target cells by monocytes that had been adhered for 30 min at 37° in RPMI was examined as described in the Materials and Methods. Phagocytosis of C3d-coated cells was similar to that of C3b and iC3b coated cells and markedly exceeded EAC14 and EAC14$_T$+IgG.

Table 3. Rosette inhibition by anti-CR1 and anti-CR3 monoclonal antibodies

	Mean percentage inhibition*				
Cell type	3D-9†	Anti-Mol‡	3D-9+ anti-Mol	Mouse IgM‡	Mouse IgG†
EAC3b	90·6±3·8* (6)	2·2±2·2 (4)	98·5±0·87 (3)	12·7±7·8 (4)	5·96±5·96 (3)
EAC3bi	10·25±10·25 (4)	59·2±9·4 (6)	65·5±14 (4)	1·03±1·03 (3)	10±3 (2)
EAC3d	6·8±2·8 (6)	42·1±12·2 (6)	62±3·9 (4)	2·9±2·3 (5)	14±14 (2)

Monocytes were adhered for 3–5 hr at 37° in the presence of 10% FCS. After three washes with RPMI, cells were incubated with the monoclonal antibodies for 30 min at 37° before the addition of red cell intermediates. Rosette formation was examined as described in the Materials and Methods.

* Mean±SE, number of experiments in parentheses.

† 3D9 and mouse IgG were added at concentrations ranging from 40–80 μg/ml.

‡ Anti-Mol and mouse IgM were added at 20–40 μg/ml.

studied using low levels of sensitizing IgG at which the synergistic effect of C3d was most prominent (~600 IgG/cell). Mol antibody at 40 μg/ml blocked ingestion of each of the IgG sensitized cellular intermediates (EAC14-IgG by 75%; EAC3b-IgG by 93%; EAC3bi-IgG by 42%; and EAC3d-IgG by 66%). Mouse IgM control antibody had no effect on phagocytosis.

The effect of EDTA on EAC3d binding to monocytes

Binding via the CR3 receptor is metal-dependent and can be blocked by EDTA (Wright & Silverstein, 1982; Ross *et al.*, 1983). EAC3d and EAC3bi rosette formation with cultured monocytes was markedly blocked in the presence of 5 mM EDTA (from 53 to 7% and from 28 to 0% for EAC3bi and EAC3d, respectively). On the other hand, EAC3b rosette formation was not changed by EDTA at concentrations as high as 20 mM (49% with EDTA, 48% without EDTA).

DISCUSSION

It is well documented that monocytes and PMN express receptors that recognize C3b and iC3b (reviewed by Ross & Medof, 1985). Early workers also reported binding of 'C3d'-coated particles to phagocytes (Ehlenburger & Nussenzweig, 1977; Welleck, Hahn & Opferkuch, 1975; Reynolds *et al.*, 1975) and even a phagocytic function for bound C3d was described (Ehlenburger & Nussenzweig, 1977). These early studies predated the discovery of the intermediate cleavage fragments of C3, iC3b and C3dg, and the target particles used at that time probably contained a mixture of the C3 fragments. However, Carlo *et al.* (1979) reported EC3d binding to monocytes with target cells that had been treated with H, I and trypsin, which should bear only the C3d fragment.

There has been renewed interest in the receptor-binding properties of the degradation products of C3, triggered by the current knowledge of C3 fragmentation, the demonstration of multiple C3 receptors that recognize different C3 fragments, and the preparation of monoclonal antibodies to these receptors and to the C3 fragments. The first reports using well-characterized C3d-coated erythrocytes as targets suggested that these targets do not bind to phagocytic cells (Beller, Springer & Schreiber, 1982; Ross & Lambris, 1982). These reports were in keeping with studies showing that C3d-coated erythrocytes have normal survival in circulation (Schreiber & Frank, 1972; Atkinson & Frank, 1974).

In 1983 we reported that rosette formation by C3d-coated sheep erythrocytes (EAC3d) to peripheral blood monocytes could be achieved by allowing the monocytes to adhere to a glass surface and incubating these cells in culture with FCS for a short time. In contrast, non-cultured monocytes bound EAC3d very poorly. A high density of C3d was required for rosette formation to occur (> 10,000/cell). Binding of EC3dg was not examined in these studies (Inada *et al.*, 1983). Since that report a number of other groups have observed that C3d or C3dg is capable of binding to phagocytic cells. Ross and co-workers showed binding of C3dg but not C3d-coated sheep cells and microspheres to phagocytes in suspension. They concluced that a CR2-like receptor was involved in C3dg binding, which they proposed occured via a binding site located in the C3d region of the molecule (Ross *et al.*, 1983). Later, Vik & Fearon (1985) showed specific binding of radiolabelled C3dg monomers and oligomers to neutrophils. They proposed that a receptor exists on neutrophils that is distinct from CR1, CR2 and CR3, which is capable of binding iC3b, C3dg and C3d. Previously CR2 had been shown to bind these three fragments of C3 (Ross *et al.*, 1983). However, anti-CR2 (HB5) did not bind to PMN in Vik's studies. The work of Frade and co-workers also supported the existence of a new C3dg-binding receptor on phagocytes, which

is distinct from CR2. Using polyclonal anti-CR2 (anti-gp 140), they showed that this antibody did not block the uptake of EC3dg on neutrophils. Anti-CR1 and anti-CR3 (MN-41, anti-α chain) monoclonal antibodies also failed to block EC3dg binding, according to Frade (1985). In related studies, Wright, Licht & Silverstein (1984) reported that EC3d could bind to cultured monocytes after stimulation of the monocytes with phorbol myristate acetate (PMA). They reported that a monoclonal antibody (IB4), which is directed against the β chain (common to LFA-1 and CR3), bound to the C3d-binding protein on monocytes and blocked EC3d rosetting. Other antibodies directed against CR3 (OKM10) and LFA-1 (TA-1) did not alter rosetting. Thus, they concluded that EC3d bound to PMA-stimulated monocytes via a CR2-like receptor, with antigenic properties in common with CR3 and LFA-1.

Here we report further studies of the C3d-binding properties of monocytes cultured for a relatively short term (3–5 hr) in FCS. Since low levels of bound C3b and iC3b can induce binding to CR1 and CR3, careful characterization of the sheep cell intermediates is critical to studies of C3d function. Several conventional procedures were followed to demonstrate that EAC3d were not contaminated with C3b or iC3b (see the Results). Using ^{125}I monoclonal antibodies directed against C3c, iC3b neo-antigen and C3d, we quantified the concentration of the C3 fragments on the cellular intermediates. C3b and iC3b were not detected on any of the EAC3d preparations used in these studies (Table 1). In contrast, as few as ~80 C3b/cell and ~50 iC3b/cell could be detected by the binding of ^{125}I-anti-C3c and ^{125}I-anti-iC3b, respectively. Contamination of EAC3d with 80 C3b or 50 iC3b would not account for the EAC3d activities reported here. SDS–PAGE analysis also showed that the EAC3d preparations did not contain the C3bγ′ or 68,000 MW fragment, which characterize C3b and iC3b, respectively. Also, there was no contaminating C3dg by SDS–PAGE analysis (not shown).

We showed that monocytes cultured in FCS formed rosettes with EAC3d, as we had previously reported (Table 1). Unlike Wright *et al.* (1984), EAC3d binding did not require stimulation of monocytes with PMA if EAC3d were coated with a high density of C3d. However, the involvement of some form of stimulation of the phagocytic cells was suggested by the fact that EAC3d did not adhere to monolayers formed in the presence of BSA rather than FCS (Inada *et al.*, 1983). This suggests that a change in the affinity or surface expression of the C3d-binding receptor occurred when monocytes were cultured in FCS.

C3d enhances rosette formation of EIgG with non-cultured monocytes (not shown). This results suggests that the C3d-binding receptor is present on non-cultured monocytes but apparently has poor affinity for target particles coated only with C3d. Importantly, C3d bound to erythrocytes was shown to have an opsonic function. Like C3b and iC3b, it enhanced the ingestion of IgG-coated cells. As few as 300 molecules of IgG, acting in concert with C3d, led to the ingestion of target cells prepared with 1 U/ml input of C3 (Table 2). Ingestion of EAC3d-IgG greatly exceeded that of EAC14$_T$-IgG and was similar to that of EAC3b-IgG and EAC3bi-IgG. Interestingly, in these experiments iC3b was a more effective opsonin than C3b on a per molecule basis. This may reflect the fact that iC3b can interact with more than one C3 receptor (CR3, CR1 and CR4) (Ross *et al.*, 1983). C3b only interacts with CR1. Interestingly, phagocytosis of EAC3d-IgG did not require incubation of monocytes with FCS and occurred with monocytes in suspension as well as with adherent cells. In the absence of IgG, C3-coated erythrocytes were not generally ingested by the monocytes, although a low level of EAC3bi ingestion was consistently observed. This finding, that C3d augmented phagocytosis of erythrocytes coated with comparatively low levels of IgG, is of great interest in view of previous clearance studies that showed normal survival of C3d/C3dg-coated erythrocytes (Schreiber & Frank, 1972; Atkinson & Frank, 1974). Since earlier clearance studies examined clearance of IgM sensitized cells, it will be interesting to determine if C3d and C3dg augment clearance of IgG sensitized erythrocytes *in vivo* as these findings suggest.

Notably, our data indicate that CR2 does not play a role in C3d binding to monocytes. On the other hand, binding of EAC3d was substantially blocked with anti-CR3 monoclonal antibody (anti-Mol) (Table 3). Anti-CR1 monoclonal antibody augmented anti-Mol inhibition to a slight but statistically significant degree, but complete inhibition was not achieved. Previous studies suggested that only iC3b can bind to CR3 (reviewed by Ross & Medof, 1985). Our results indicate that CR3 is also capable of binding EAC3d. However, the affinity of C3d binding to CR3 is apparently low, as indicated by the requirement of a high density of C3d for rosette formation with cultured monocytes and the lack of C3d rosetting with non-cultured monocytes. Thus, the interaction of C3d with monocyte CR3 would not be detected in many assay systems. CR1 may also contribute to the binding of EAC3d to a small extent. The fact that anti-CR1 plus anti-CR3 did not completely block EAC3bi or EAC3d rosetting raises the possibility that the recently described receptor (CR4) may facilitate binding of iC3b as well as C3d to cultured monocytes. Since it is often observed that anti-CR monoclonal antibodies do not inhibit receptor function completely, this finding may simply reflect incomplete blockade of the binding site by the monoclonal antibodies. The importance of CR3 to C3d binding is further supported by the striking inhibitory effect of EDTA on EAC3d rosette formation. This suggests that the C3d-binding receptor under study here is different from the neutrophil C3dg receptor described by Vik, which is not EDTA sensitive (Vik & Fearon, 1985).

We also found that the ingestion of EAC3d-IgG was greatly reduced when monocytes were preincubated with anti-Mol antibody. However, a similar inhibitory effect was observed with IgG-coated EAC14b, EAC3b and EAC3bi, suggesting that anti-Mol blocks ingestion by direct interference with the phagocytic process rather than by specifically blocking CR3. This finding is in agreement with Anaout *et al.* (1983).

In summary, our findings indicate that CR3 is primarily responsible for the binding of C3d to monocytes. The interaction of C3d with CR3 on non-cultured peripheral blood monocytes is of such low affinity that rosettes are not formed except when C3d coated erythrocytes are sensitized with low levels of IgG. After a relatively short time in culture with FCS, monocytes are capable of forming rosettes with EAC3d, suggesting that the function or surface expression of CR3 is under some form of physiological regulation. The interaction of CR3 with C3d may be of importance as a host defence mechanism since C3d acts in synergy with IgG to augment FcR-mediated phagocytosis.

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