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Immunology

C3d receptors are expressed on human monocytes after *in vitro* cultivation

(complement/Raji cell/monocyte/complement receptor)

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ABSTRACT **Highly purified human third component of complement (C3) was used to coat sheep erythrocytes (E) that were sensitized with IgM antibody (EA), forming EAC3b over a wide range of C3 molecules per cell. EAC3b were converted to EAC3bi by incubation with purified C3b inactivator (factor I) and β1H globulin (factor H). EAC3bi were in turn trypsinized to produce the cellular intermediate EAC3d. Each of the cell types was carefully characterized to be certain of the type of C3 determinant expressed. These cellular complement intermediates were used to assess by rosette formation the C3 receptor activity on peripheral blood monocytes under various experimental conditions. Uncultivated monocytes from peripheral blood bound EAC3b and EAC3bi well but did not bind EAC3d significantly. However, upon cultivation on glass surfaces in the presence of fetal calf serum but not bovine serum albumin, monocytes showed a progressive increase in expression of the C3d receptor. The Fab' fragment of anti-C3c blocked binding of EAC3b completely, blocked EAC3bi partially, but failed to block binding of EAC3d to cultivated monocytes. In contrast, the Fab' fragment of anti-C3d blocked EAC3d rosette formation completely. These studies demonstrate that monocytes are capable of expressing receptor activity for a determinant on C3d but that the expression of this receptor depends on the state of activation or differentiation of the cells.**

It is well documented that receptors for immunoglobulin and complement components exist on phagocytic and lymphoid cells (1–7). These receptors have been shown to be important for normal host defense and immunologic function (8–13). Recent work has demonstrated the existence of three distinct receptors for the third component of complement (C3) designated CR_1, CR_2, and CR_3 (14). CR_1 has greatest affinity for C3b, a 185,000-dalton product of enzymatic cleavage of C3 by either C4b2a or C3bBb; CR_3 has specificity for C3bi, a hemolytically inactive form of the C3 molecule generated by the action of C3b inactivator (factor I) and β1H globulin (factor H) on C3b; and CR_2 has apparent specificity for C3d, a 30,000-dalton fragment of C3 that remains bound to the complement-activating surface when C3bi is further cleaved and the 145,000-dalton C3c is released into the fluid phase. Various phagocytic and immunologic cells are known to express different cell-surface C3 receptors. For instance, normal B lymphocytes express all three receptors, whereas the Raji lymphoblastoid cell lines are reported to express only CR_2 and CR_3 (6). Recent work has suggested that normal mature peripheral blood polymorphonuclear neutrophils (PMNs) and monocytes express only CR_1 and CR_3 (5, 15, 16). In the present work, we show that peripheral blood monocytes also can be induced to express CR_2 by short-term cultivation *in vitro* in the presence of fetal calf serum. This implies that expression of receptors for different forms of the C3 molecule may vary not only among cell types but also during the activation and differentiation of a single cell.

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MATERIALS AND METHODS

Buffers and Media. Buffers included isotonic Veronal-buffered saline (VBS); isotonic VBS without added divalent cations containing 10 mM Na_2EDTA and 0.1% gelatin; isotonic low-ionic-strength dextrose/Veronal-buffered saline (DVBS) containing 10 mM EDTA (pH 6.0) and 0.1% gelatin (1.8 mS, 0°C); and DVBS containing gelatin, Mg^{2+}, and Ca^{2+} (3.1 mS, 0°C) ($DVBS^{2+}$). All were prepared as described (17). Hanks' balanced salt solution (HBSS) with or without added divalent cations and RPMI 1640 medium with L-glutamine were purchased from GIBCO.

Reagents. Partially purified guinea pig complement component C1 and the human component C2 were purchased from Cordis (Miami, FL). Phenylmethylsulfonyl fluoride ($PhMeSO_2F$) and bovine serum albumin were purchased from Sigma. A stock solution of 0.1 M $PhMeSO_2F$ in anhydrous isopropanol was prepared just before use. Pepsin (Boehringer Mannheim), bovine pancreatic trypsin (type III, recrystallized twice), and soy bean trypsin inhibitor (type I-S) were purchased from Sigma and were used without further purification. Sepharose, Sephadex, and Sephacel products were obtained from Pharmacia (Piscataway, NJ). Ficoll/Hypaque (lymphocyte separation medium) was obtained from Bionetics (Kensington, MD), and Percoll was purchased from Pharmacia (Uppsala, Sweden). Dithiothreitol was obtained from Bio-Rad. Fetal calf serum was obtained from GIBCO. Bovine serum albumin, obtained from Sigma, was crystallized and was free of protease activity as assayed by the Bio-Rad protease detection kit.

Purification of Components. Human complement components C3 and C4 were purified from fresh human plasma by the method of Hammer *et al.* (18). Factors H and I (17) and bovine conglutinin were obtained as described (19). Antisera specifically reactive with human C3c and with human C3d were raised by immunizing sheep with highly purified C3c and C3d, which were generated by elastase cleavage of C3b. The anti-C3c reacted strongly with native C3 and C3c on immunoelectrophoretic analysis and did not react with C3d, as evidenced by failure to form a line with C3d on immunoelectrophoresis and failure to agglutinate erythrocytes coated with C3d. The anti-C3d re-

Abbreviations: C1, C2, C3, and C4, first, second, third, and fourth components of complement; VBS, Veronal-buffered saline; DVBS, low-ionic-strength (1.8 mS) dextrose/Veronal buffered-saline; $DVBS^{2+}$, DVBS containing Mg^{2+} and Ca^{2+}; E, sheep erythrocytes; EA, sheep erythrocytes sensitized with IgM antibody; EAC, complement-coated EA; HBSS, Hanks' balanced salt solution; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; PMN, polymorphonuclear neutrophil.

acted strongly with C3d in aged serum. Minimal contamination with anti-C3c was noted. The IgG fraction of each was prepared by octanoic acid precipitation (20). The $F(ab')_2$ fragment of each IgG was prepared by pepsin digestion and column chromatography on Sephadex G-150 under conditions as described (21). $F(ab')_2$ were further purified by absorption with protein A-agarose. Monovalent Fab' was obtained from the $F(ab')_2$ fragment by reduction with dithiothreitol and alkylation by iodoacetamide (22).

Preparation of Radiolabeled C3. Highly purified human C3 was radiolabeled with ^{125}I (^{125}I-C3) by a modification of the method of Bolton and Hunter (23). The specific activity of the ^{125}I-C3 preparations was 0.017 μCi/μg of C3 (1 Ci = 3.7×10^{10} Bq). Radiolabeled C3 had >90% of the specific hemolytic activity of unlabeled C3.

Cellular Intermediates. Sheep erythrocytes (E) sensitized with IgM antibody (EA) were coated with complement (EAC) to form EAC1 and EAC14 as described (24). The 25 hemolytic units of C4 used in preparation of EAC14 was less than the quantity required to mediate binding to the human erythrocyte immune adherence receptor. EAC1423b were prepared by incubating EAC14 in $DVBS^{2+}$ with human C2 (100 units/ml) and various amounts of C3 for 45 min at 30°C. In some experiments, to quantitate C3 uptake onto these cells, ^{125}I-C3 was used. After the cells were washed in $DVBS^{2+}$, EAC1423b were incubated in 1.8 mS VBS containing 10 mM EDTA for 30 min at 30°C and then incubated in $DVBS^{2+}$ at 4°C overnight to remove C1 and to allow C2 to decay. For purposes of simplicity, the resulting intermediate, with both C4b and C3b bound to the erythrocyte surface, is designated as EAC3b because it appears to bind to receptors by virtue of its surface-bound C3b. EAC3b were washed and incubated with factor I (1:200 dilution) and factor H (6.7 μg/ml) in 1.8 mS DVBS containing 10 mM EDTA for 2 hr at 37°C to convert surface-bound C3b to C3bi (EAC3bi). An aliquot of EAC3bi was washed three times with $DVBS^{2+}$ and incubated with trypsin (1.25 μg/ml) for 30 min at 37°C to prepare the cellular intermediate, EAC3d (17). At the end of this period of incubation, soybean trypsin inhibitor was added at a concentration of 2.5 μg/ml; the cells were incubated an additional 10 min and were then washed three times with $DVBS^{2+}$ at 4°C. The various cellular intermediates including E, EA, EAC14, EAC3b, EAC3bi, and EAC3d were maintained in $DVBS^{2+}$ at 4°C at a concentration of 1.5×10^8 cells per ml until use.

Complement Receptor-Positive Cells. Human type O Rho(D)-positive erythrocytes were obtained from heparin-treated venous blood. These erythrocytes were washed with VBS and then suspended and standardized at 6.5×10^7 cells per ml in $DVBS^{2+}$.

Human PMNs were isolated from heparin-treated peripheral blood of normal volunteers by centrifugation through Ficoll/Hypaque density gradients. Erythrocytes were removed by dextran sedimentation and two or three cycles of hypotonic lysis. Viability as assessed by trypan blue dye exclusion was >95%. PMNs were then washed with and stored in cold HBSS. Human monocytes were isolated by two step-density gradients of Ficoll/Hypaque and Percoll (25). Human monocytes were also prepared with a J221 centrifuge (Beckman) equipped with a JE6 elutriator rotor with two Sanderson separation chambers by following a procedure modified from Lionetti *et al.* (26). The purity of both monocyte preparations was >90% as determined by nonspecific esterase staining (Technicon) and by morphology. PMNs and monocytes were maintained in an ice bath until use.

Complement Receptor and Rosette Inhibition Assay. Assays were performed on cells in suspension and, where noted, on cells that had been allowed to adhere to glass plates. For assays of cells in suspension, 50 μl of cells in HBSS without divalent cations were suspended at 2×10^6 cells per ml in 12 × 75 mm polypropylene tubes and were then mixed with 50 μl of either E or the various EAC preparations in $DVBS^{2+}$ and centrifuged 5 min at 50 × *g*. After 30 min of incubation of the mixture in a 5% CO_2 incubator at 37°C without mixing, the mixtures were gently agitated and examined under the light microscope. In assays of monocyte monolayers, 250 μl of monocytes in RPMI 1640 medium plus 10% fetal calf serum at a concentration of 2×10^5 cells per ml were added to each of the eight chambers of a slide-mounted tissue culture plate (Lab-Tek, Naperville, IL) and incubated for the indicated time in a 5% CO_2 incubator. After the plates were washed two times with HBSS, the chambers were overlaid with medium and various cellular intermediates as receptor probes. The plates were then centrifuged at 50 × *g* for 5 min and incubated as described above. After incubation, the chambers were washed two times with HBSS, fixed with 0.5% glutaraldehyde, and stained with Giemsa stain. In experiments where indicated, 1% bovine serum albumin was substituted for fetal calf serum during the monocyte monolayer assay.

In rosette inhibition assays, indicator probes were preincubated for 30 min at 37°C before the rosette assay was per-

Table 1. Characterization of cellular intermediates

Property	C3 molecules × 10^{-2} per cell			
	Immune adherence			
Cellular intermediates	3.6	36	120	240
EAC3b	3+*	4+	4+	4+
EAC3bi	3+	3+	3+	3+
EAC3d	0	0	0	0
	Conglutination			
Cellular intermediates	3.6	36	120	240
EAC3b	−†	−	−	−
EAC3bi	+	+	+	+
EAC3d	−	−	−	−

*The settled cells were scored visually from 0 to 4+ depending on the degree of adherence activity as described (17).
†Agglutination by conglutinin was scored − or + after incubation.

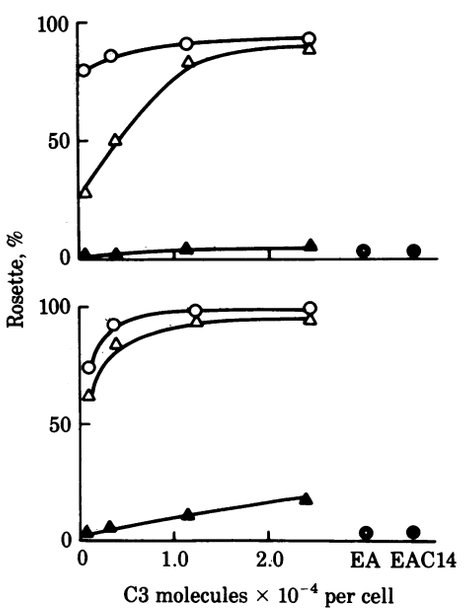

FIG. 1. Quantitative and qualitative analysis of C3 receptors of PMNs (*Upper*) and noncultivated monocytes (*Lower*): EAC3b (○), EAC3bi (△), EAC3d (▲), EA (◎), and EAC14 (◉).

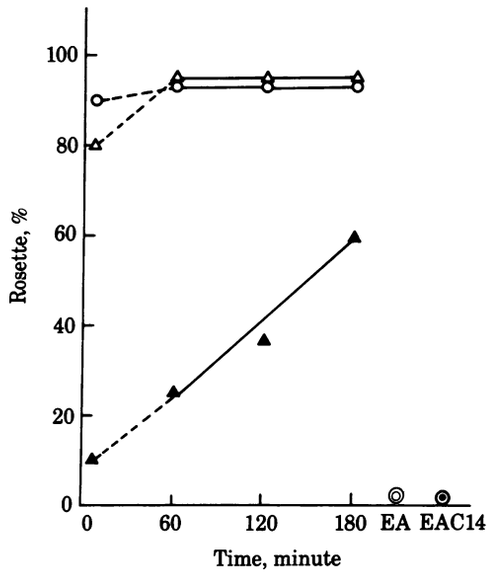

FIG. 2. C3d receptors of cultivated monocytes. EAC3d rosettes with monocytes increased during cultivation. ○, EAC3b; △, EAC3bi; ▲, EAC3d. All cells (C3b, C3bi, C3d) had 1.2 × 10^4 C3 molecules per E. Time zero means fluid-phase assay.

formed with a dilution of Fab' anti-C3c chosen to produce 100% inhibition of EAC3b rosettes. In both types of assays, test cells with three or more adherent E under the light microscope at ×400 magnification were scored as complement-receptor positive. All assays were done in buffers containing 1 mM $PhMeSO_2F$ to prevent the action of leukocyte proteases on C3b and C3bi (27).

Immune Adherence, Conglutination, and Antibody Hemagglutination Reactions. The immune adherence reaction was performed as described (28). Briefly, various EACs, suspended in DVBS to 1.5 × 10^8 cells per ml, were added to an equal volume of human erythrocytes (6.5 × 10^7 cells per ml) in a microtiter tray. After mixing, the reactants were allowed to settle during a 10-min incubation at 37°C. The settled cells were then scored visually from 0 to 4+ depending on the degree of adherence activity observed.

For conglutination assays, 50 μl of purified bovine conglutinin (6.5 μg/ml) was added to 50 μl of various cellular complement intermediates in $DVBS^{2+}$ in a microtiter tray. After mixing well and incubating the reactants for 30 min at room temperature, the degree of agglutination of the settled cells was scored visually. For antibody hemagglutination tests, 50 μl of serially diluted anti-C3c-IgG was incubated with the cellular intermediates, and the agglutination was scored after 30 min at room temperature.

RESULTS

Characterization of Cellular Intermediates. Cellular C3 intermediates were prepared over C3 concentrations ranging from 360 to 240,000 molecules bound per cell. The number of C3 molecules per E was calculated based on the uptake of radiolabeled ^{125}I-C3. EAC3b was converted to EAC3bi or EAC3d as described. At all concentrations of added C3, EAC3b and EAC3bi were positive in immune adherence (Table 1). However, only EAC3bi were positive for conglutination. EAC3d cells were negative in both immune adherence and conglutination assays. Moreover, unlike EAC3b and EAC3bi, the cells were not agglutinated by anti-C3c antibody. E, EA, and EAC14 were all negative for immune adherence, conglutination, and anti-C3c antibody hemagglutination.

Quantitative and Qualitative Analysis of C3 Receptors on Phagocytic Cells. Both PMNs (Fig. 1 *Upper*) and monocytes (Fig. 1 *Lower*) bound EAC3b. At a low input of C3, PMNs did not bind EAC3bi as avidly as monocytes even in the presence of 1 mM $PhMeSO_2F$. PMNs did not bind the EAC3d indicator particles; however, the monocyte preparation consistently formed a minimal number of rosettes with EAC3d when indicator particles bearing large numbers of C3d molecules were used.

C3d Receptors of Cultivated Monocytes. The reactivity of cultivated monocytes with EAC3d increased dramatically. Fig. 2 shows that monocyte rosettes increased from 9% without cultivation to 25% after 1 hr, to 34% after 2 hr, and to 59% after 3 hr of cultivation at 37°C using indicator cells bearing 1.2 × 10^4 C3 molecules per E. In contrast, the reactivity of monocytes with EAC3b and EAC3bi did not change markedly. Upon cultivation, neither E, EA, nor EAC14 bound to monocytes.

C3d Receptor Expression by Monocytes Cultured in Fetal Calf Serum and Bovine Serum Albumin. To examine the requirements for monocyte expression of C3d receptors, monocyte monolayers were prepared either in RPMI medium containing 10% fetal calf serum or, alternatively, in 1% bovine serum albumin. Equal numbers of cells bound to glass in the two media; equivalent cell spreading was apparent, and monocyte monolayers expressed C3b and C3bi receptors in both media.

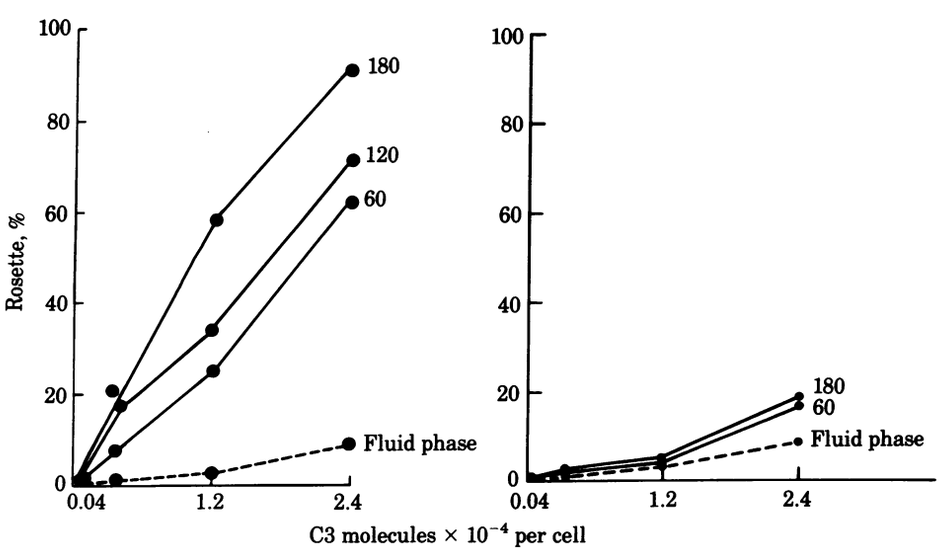

FIG. 3. C3d receptor expression by monocytes cultured in fetal calf serum and bovine serum albumin. ——, EAC3d binding to cultivated monolayer monocytes incubated with either 10% fetal calf serum (*Left*) or 1% bovine serum albumin (*Right*) from 60 to 180 min; – – –, EAC3d binding to noncultivated monocytes.

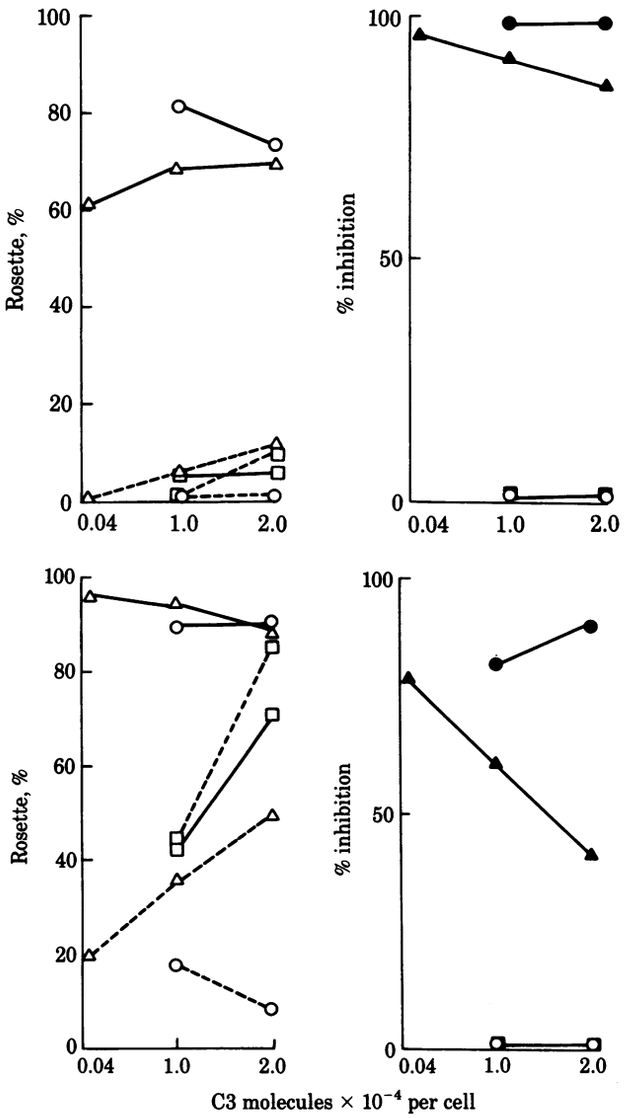

FIG. 4. Inhibition of various C3 receptors on monocytes by Fab' fragment of anti-C3c antibody. (*Upper*) Noncultivated suspension monocytes. (*Lower*) Cultivated 3-hr monolayer monocytes. ○——○, EAC3b; ○---○, EAC3b inhibition by Fab'; △——△, EAC3bi; △---△, EAC3bi inhibition by Fab'; □——□, EAC3d; □---□, EAC3d inhibition by Fab'; ●, % inhibition of EAC3b; ▲, % inhibition of EAC3bi; □, % inhibition of EAC3d.

However, only cells that adhered to glass in the presence of fetal calf serum displayed C3d-receptor activity. Fig. 3 *Left* shows that monocyte rosettes increased from 9% without cultivation to 65% after 1 hr, to 72% after 2 hr, and to 92% after 3 hr of cultivation at 37°C by using indicator particles with 2.4×10^4 C3 molecules per E. Fig. 3 *Right* shows that, in contrast, monocyte monolayers cultured in bovine serum albumin did not bind EAC3d cells well. This difference between monolayers cultured in albumin and fetal calf serum was apparent as early as 1 hr of incubation. To examine the fetal calf serum requirement in greater detail, monocytes that adhered to glass in the presence of either fetal calf serum or albumin were washed, and EAC3d cells were then incubated with the monolayers in the alternative media. Only when fetal calf serum was present during the adherence process were C3d receptors expressed.

Inhibition by Fab' Anti-C3c and Fab' Anti-C3d. In order to determine whether the binding of EAC3d to cultured monocytes was the result of residual uncleaved C3b or C3bi on the erythrocytes surface, the ability of Fab' anti-C3c and anti-C3d to block rosettes was examined. As shown in Fig. 4 *Upper*, Fab' anti-C3c completely blocked EAC3b rosettes with noncultivated monocytes and markedly inhibited EAC3bi rosettes in suspension assays. As shown in Fig. 4 *Lower*, Fab' anti-C3c almost completely blocked EAC3b rosettes, which were formed with cultivated monocytes as well, and markedly blocked EAC3bi rosettes in the monolayer assay. Fab' anti-C3c did not inhibit EAC3d rosettes at all. In experiments not shown in the figure, binding of EAC3d prepared with 1,000 C3 hemolytic units was reduced from 48% to 5% by preincubation of the EAC3d with anti-C3d. Thus, EAC3d rosetting to cultured monocytes could not be attributed to residual uncleaved C3b or C3bi.

DISCUSSION

In 1968, Gigli and Nelson reported that phagocytic cells had membrane receptors for the complement component C3 and that this component was responsible for opsonization of foreign particles (1). Later, it was shown by Nussenzweig and his colleagues that lymphocytes have C3 receptors as well (9). Since that time, the biological function of these receptors has been under intensive study in a number of laboratories (10–13). It was shown that receptors existed not only for C3b (CR_1) but also for various cleavage fragments of C3b including C3bi (CR_3) and C3d (CR_2).

It is generally agreed that monocytes and PMN have receptors for C3b and C3bi (6, 7, 29). However, the presence of receptors for C3d on phagocytic cells has been in doubt. Although early workers demonstrated that monocyte and macrophages expressed C3d receptors, the intermediate cleavage product C3bi was unknown at that time (30–32). With better understanding of the C3 cleavage pathway, it was possible to show that cellular component intermediates with C3b cleaved by the action of either serum or factors H and I often have C3bi and C3d on their surface.

Many recent studies on complement receptors have suggested that monocytes do not have C3d receptors and that earlier studies that suggested the presence of C3d receptors may have been positive because the probes used had C3bi as well as C3d on their surface. However, a number of observations are not consistent with that formulation. For instance, Carlo *et al.* suggested that C3b-coated erythrocytes treated with factors H and I and with trypsin, which should not have surface-bound C3bi, were able to bind human monocytes through their C3d receptors (33). Additionally, Ehlenberger and Nussenzweig suggested that C3d on the surface of a particle coated with suboptimal amounts of IgG augmented monocyte phagocytosis of that particle (32). Although they did not exclude the presence of C3bi molecules on the indicator particles, cellular intermediates were prepared in a way that made the presence of large numbers of C3bi molecules unlikely. Thus, it seemed appropriate to reinvestigate the nature of the complement receptors on monocytes by using carefully characterized intermediates bearing C3b, C3bi, and C3d. Our studies with these intermediates show that freshly isolated fluid-phase monocytes bind minimally to EAC3d. Although binding of EAC3d to up to 10% of the monocytes was seen, low-level contamination by lymphocytes (which are known to bear C3d receptors) could not be excluded as the cause of the observed rosetting. However, cultivation for as short a period as 1 hr on glass plates dramatically increases the number of monocytes rosetting with C3d-coated erythrocytes. Moreover, demonstration of this rosetting is dependent on the number of C3d molecules on the indicator particles. A higher density of C3d is required to demonstrate

rosetting with cultured monocytes than is required for EAC3d rosette formation with Raji cells (unpublished data). The indicator cells bearing C3d were shown to be free of C3bi and C3b by a number of methods. They were immune-adherence negative. They did not bind bovine conglutinin (unlike C3bi-coated cells) and they could not be agglutinated by anti-C3c, but they were agglutinated by anti-C3d. Most important, their rosetting was not inhibited at all by Fab' anti-C3c which was shown to inhibit C3b binding totally and C3bi binding partially. Anti-C3d, however, blocked rosettes almost completely.

There are a number of important methodologic differences between our studies and those of other investigators who have not found a monocyte C3d receptor. First, all of our studies were performed with careful dose–response control over the quantity of C3 on the indicator erythrocytes. Second, phagocytic cells were studied in suspension as well as after adherence to a glass or plastic surface. In the studies of Ross *et al.* (34), cells were examined in suspension only. In those of Ehlenberger and Nussenzweig (32), who first reported the presence of a monocyte C3d receptor, adherent cells were examined. Third, rosetting was performed in a low-ionic-strength buffer which increases receptor binding. Finally, our studies were performed in buffers containing fetal calf serum rather than bovine serum albumin, which was used by other investigators (5). The reason for the differences between fetal calf serum-cultured cells and bovine serum albumin-cultured cells is unknown. It may be that contaminants in the albumin influence the presence or expression of the receptors. However, monocytes are known to undergo a number of metabolic and cell-surface changes on adherence to glass and during cultivation in fetal calf serum-containing media, which may occur as late as 3–5 days after beginning *in vitro* cultivation (35). A likely possibility is the expression of C3d receptors by monocytes requires not only cultivation on glass but also other environmental factors which are present in fetal calf serum but not in an equivalent protein concentration of bovine serum albumin.

C3bi binding to monocytes is said to occur by a receptor distinct from CR_1 and CR_2. Because EAC3d do not bind well to fluid-phase monocytes, it is probable that C3bi binding to these cells occurs by a third C3 receptor, CR_3. Interestingly, anti-C3c can totally abrogate the binding of EAC3bi to fluid phase but not to glass-adherent monocytes. This suggests that C3bi binds to CR_3 by a determinant on or near the C3c region of the molecule but may also bind the CR_2 of cultivated monocytes through a determinant in the C3d region of the molecule.

The nature of the monocyte C3d receptor, the consequences of receptor binding, and the signal for receptor induction are all unknown at the present time. Clearly, however, the use of careful dose–response analysis of receptor binding has led to new insights. Further studies will elucidate the biology, immunochemistry, and physiology of the monocyte C3d receptors.

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