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COMPLEMENT RECEPTOR TYPE THREE-DEPENDENT DEGRADATION OF OPSONIZED ERYTHROCYTES BY MOUSE MACROPHAGES[1]

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The role of the complement receptor type 3 (CR3) on thioglycollate-elicited peritoneal macrophages (TG-PM) in the destruction of opsonized particles was studied. We found that sheep red blood cells (E) that were opsonized with an IgM monoclonal anti-Forssman antibody and complement (E-IgM-C) were lysed by TG-PM, whereas there was little lysis of E pretreated with either the antibody or the complement source alone. Furthermore, this lysis could be inhibited by anti-CR3 monoclonal antibodies that had previously been shown to inhibit binding of E-IgM-C to the CR3. Kinetic studies of phagocytosis and lysis indicated that lysis of E-IgM-C occurs after phagocytosis, suggesting that lysis is an intracellular event. Further findings suggested that intracellular lysis was promoted by CR3 bound to the phagocytosed target, because a monoclonal anti-CR3 antibody decreased the rate of phagocytosis of E-IgM-C but not its magnitude, whereas the rate and extent of lysis were strikingly inhibited. Furthermore, TG-PM that had already internalized unopsonized E selectively lysed E-IgM-C that were added later. These data confirm that the interaction of the CR3 with its ligand on E-IgM-C promotes rapid phagocytosis, and further suggest that the CR3 facilitates degradation of the target particle once internalization has occurred.

Macrophages bear two receptors for the third component of complement (C),[3] C3. The C receptors type 1 (CR1) and 3 (CR3) demonstrate preference for the C3b and iC3b ligands, respectively (reviewed in Reference 1). Study of these receptors has been facilitated by antibodies specific for the mouse and human CR3 (2, 3) and for the human CR1 (4–6). Macrophages can adhere via these receptors to particles appropriately opsonized with C. Furthermore, macrophages stimulated with fibronectin (7), phorbol ester (8), or certain lymphokines (9), or elicited with thioglycollate broth (10), can phagocytose C3-opsonized targets. In contrast, blood monocytes and resident peritoneal macrophages are competent for adherence but not for phagocytosis. C3 receptors have been shown to interact synergistically with Fc receptors in enhancing the extracellular lysis and phagocytosis of IgG and C3-treated targets (11–15) by polymorphonuclear cells, macrophages, and lymphocytes. However, there have been no reports showing that complement receptors are able to independently mediate lysis of C-coated targets. Furthermore, the effects of C receptors on the fate of particles subsequent to ingestion have not been reported.

In this paper, we have characterized particle destruction mediated by the mouse macrophage CR3. We report that phagocytosis by thioglycolate-elicited peritoneal macrophages (TG-PM) of C3-opsonized sheep erythrocytes (E) via the CR3 leads to a relatively rapid degradation of the opsonized E. Furthermore, we show that the degree of lysis of C3-opsonized E by TG-PM is greatly reduced by the presence of monoclonal antibodies (MAb), which block the function of the CR3, whereas the rate of phagocytosis of these E is only slightly inhibited.

MATERIALS AND METHODS

Mice. C57BL/6 mice, ages 6 to 20 wk, were used as a source of macrophages. Sera from A/J mice (The Jackson Laboratory, Bar Harbor, ME) were used as a source of C5-deficient C.

Macrophages. Peritoneal macrophages were obtained from C57BL/6 mice that were given 1 ml of 3× thioglycollate medium i.p. 3 to 4 days before lavage with Hanks' balanced salt solution containing 5 mM HEPES buffer (H/H).

Preparation of E. Sheep red blood cells (E) (Colorado Serum Co., Denver, CO) were stored in Alsever's solution and washed three times with H/H before use. E (3×10^8 in 100 μl H/H) were labeled by incubation with 100 μCi of $Na_2{}^{51}CrO_4$ in 100 to 200 μl of saline (New England Nuclear, Boston, MA) for 2 to 3 hr at 37°C with occasional shaking. E were washed three more times and resuspended to 10^7 E/ml. ^{51}Cr-E were then incubated at room temperature for 30 min with a 1/1000 dilution of heat-inactivated M1/87 MAb supernatant (a monoclonal rat IgM anti-Forssman antibody) (16), a 1/1000 dilution of IgG fraction of rabbit anti-E antiserum (Cordis Laboratories, Inc., Miami, FL), or H/H alone. Cells were then washed twice with H/H, resuspended to 3×10^7 E/ml, and incubated for 45 min at 37°C either alone or with a 1/70 dilution of E-absorbed A/J mouse serum. E were then washed twice more and resuspended to appropriate concentrations with RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 50 μg gentamicin/ml, and 2 mM glutamine (complete medium).

Cytotoxic and phagocytic assay. Cytotoxicity assays were performed as described (17). Briefly, 10^5 thrice-washed PM in 100 μl of complete medium were added to wells of a flat-bottomed microtiter plate (Costar No. 3596) containing 50 μl of complete medium and, where appropriate, MAb. After 30 min, 2×10^5 ^{51}Cr-labeled opsonized or unopsonized E in 50 μl of complete medium were added to the wells, and the cells were incubated for 18 hr unless otherwise stated. Cytotoxicity assays were terminated by collecting 100 μl of supernatant and counting it in a well-type gamma counter. All assays were done in triplicate.

To measure phagocytic activity, wells were first washed with H/H to remove excess released ^{51}Cr. Intact, non-internalized E were then lysed with distilled H_2O for 15 sec at room temperature, followed by

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[3] Abbreviations used in this paper: E, sheep red blood cell; TG-PM, thioglycollate-elicited peritoneal macrophage; C, complement; CR3, C receptor type 3; MAb, monoclonal antibody; E-IgM, E sensitized with IgM antibody; E-IgM-C, E-IgM sensitized with serum from A/J mice as a source of C; E-IgG, E sensitized with IgG; E:T, effector to target ratio.

two washes with H/H. Macrophages were then lysed by incubation with 200 μl of 0.5% Nonidet P-40 overnight at 37°C; 100 μl of lysate were collected for counting in a well-type gamma counter. All assays were done in triplicate.

Calculation of cytotoxic or phagocytic activity was done as follows:

$$\% \text{ Specific lysis (phagocytosis)} = \frac{E - C}{T - C} \times 100$$

where T is the amount of ^{51}Cr released when ^{51}Cr-E are incubated with 0.5% Nonidet P-40, E is the amount of release in experimental groups, and C is the amount of control ^{51}Cr release from ^{51}Cr-E incubated alone for the duration of the assay (or ^{51}Cr remaining in wells having no peritoneal macrophages after the lysis and washing protocol for phagocytic assays). Input ^{51}Cr per well was in the range of 6000 to 9000 cpm, and spontaneous release did not exceed 15% of input counts.

RESULTS

The fate of E that were sensitized with IgM and C alone or together and cultured for 18 hr with TG-PM was examined (Fig. 1). There was very little lysis of E that were presensitized only with anti-Forssman IgM MAb (E-IgM) or only with E-absorbed, C5-deficient A/J mouse serum as source of C. However, there was a much higher degree of lysis by TG-PM when E were pretreated sequentially with anti-Forssman IgM and A/J serum (E-IgM-C). Although E and E-IgM preparations were not efficiently lysed, they were markedly phagocytosed by TG-PM at the 18-hr time point. These data suggest a synergistic role for IgM anti-E antibodies and A/J serum in mediating the lysis of E by TG-PM.

Role of C in mediating E lysis by TG-PM. Several approaches were used to show that C in A/J serum was responsible for rendering the E-IgM susceptible to TG-PM lysis. E-IgM treated with A/J serum that had been heat inactivated by incubation at 56°C for 30 min to prevent deposition of C3 fragments on the E membrane were cultured with TG-PM for 18 hr. It was found that although there was considerable lysis of E treated with IgM + fresh A/J serum, the amount of lysis of E-IgM + heat-inactivated A/J serum was the same as the control values of E alone or E-IgM (Table I), thus suggesting that C is able to opsonize E-IgM for lysis by TG-PM.

An anti-CR3 MAb was used to further examine the role of C and macrophage C receptors in this lytic reaction. Under the conditions used here for opsonization with C5-deficient A/J serum, the preponderance of C3 deposited on the red cell surface is converted to iC3b (18). The macrophage CR3 has previously been demonstrated to

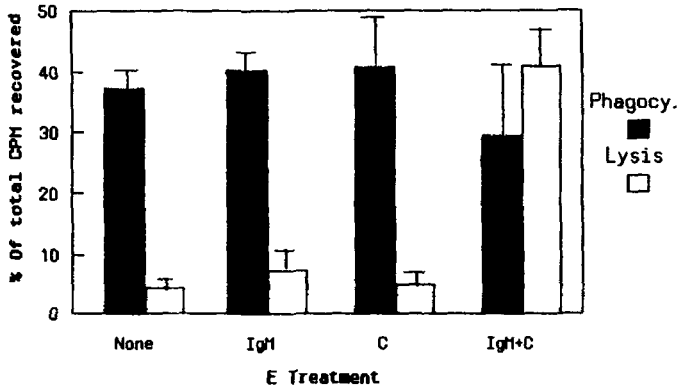

Figure 1. Ability of TG-PM to internalize (*solid bars*) and lyse (*open bars*), E, E-IgM, E + C, or E-IgM-C in an 18-hr ^{51}Cr-release assay. Each bar represents the average of at least seven experiments for cytotoxicity and four experiments for internalization.

TABLE I

Effect of heat inactivation of A/J serum on lysis of E-IgM + A/J serum by TG-PM

Expt.	Percent Specific ^{51}Cr Release[a]			
	E	E-IgM	E-IgM$^+$ A/J Serum	E-IgM$^+$ Heat-inact. A/J Serum[b]
1	4.6	8.5	39.0	5.6
2	3.9	5.2	39.7	2.7
3	3.8	6.3	34.1	4.1
4	3.3	8.9	37.8	4.9

[a] Eighteen-hour ^{51}Cr-release assay with an effector to target ratio (E:T) of 1:2.

[b] A/J serum was heated at 56°C for 30 min.

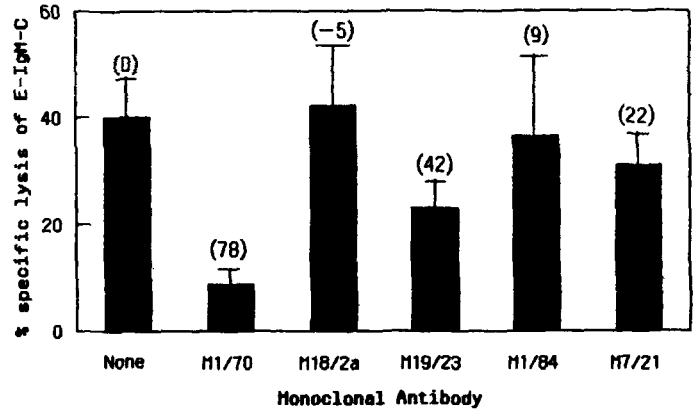

Figure 2. Inhibition by monoclonal antibodies of CR3-dependent lysis. MAb that inhibit rosetting of E-IgM-C to TG-PM via the CR3 (M1/70 and M19/23). MAb that bind to the CR3 but do not inhibit rosetting (M18/2a), MAb that are anti-pan-leukocyte (M1/84), and MAb that are anti-H-2 (M7/21) were added to macrophages immediately before addition of ^{51}Cr-E-IgM-C (E:T-1:2). ^{51}Cr-release was measured after 18 hr. Percent inhibition of ^{51}Cr-release (*in parentheses*) and standard deviation are shown. Each bar represents a minimum of five experiments.

mediate adherence to iC3b opsonized particles (2). M1/70, a rat IgG2b MAb to the CR3, was therefore tested for inhibition of lysis. M1/70 was indeed able to inhibit lysis of E-IgM-C by almost 80% (Fig. 2). M19/23, another rat IgG2b monoclonal antibody that reacts with the α-chain of the CR3 and is able to inhibit rosetting of E-IgM-C to TG-PM (19) was also able to inhibit lysis of E-IgM-C, although to a lesser degree. M18/2a, on the other hand, a rat IgG2a MAb that binds to the β-chain of the CR3 and does not block E-IgM-C rosetting (19), had no effect on TG-PM lysis of C-coated E. Further specificity controls showed that M1/84 (an IgG2a) and M7/21 (an IgG2b), two monoclonals produced in rats with anti-H-2 and pan-leukocyte reactivity, respectively, had only small inhibitory effects on lysis of E-IgM-C. Thus these data show that the lysis observed when E are opsonized with IgM anti-E + A/J serum is due to the interaction of C3 deposited on the E with the CR3 on the TG-PM.

Effect of F(ab')$_2$ of M1/70 on lysis of E-IgM-C and E-IgG. To determine whether M1/70 inhibited other cytotoxic functions of TG-PM or specifically inhibited cytotoxicity mediated via the CR3, F(ab')$_2$ fragments of M1/70 were examined for their effect on Fc receptor-mediated lysis of E sensitized with IgG (E-IgG) as well as lysis mediated by the CR3. It was found that the F(ab')$_2$ fragments of M1/70 inhibited lysis of E-IgM-C in a dose-dependent manner but had no effect on the lysis of E-IgG at the highest concentration tested (Fig. 3). Intact M1/70 IgG inhibited lysis of E-IgG, as well as E-IgM-C, presumably by competing for the Fc receptor on the TG-

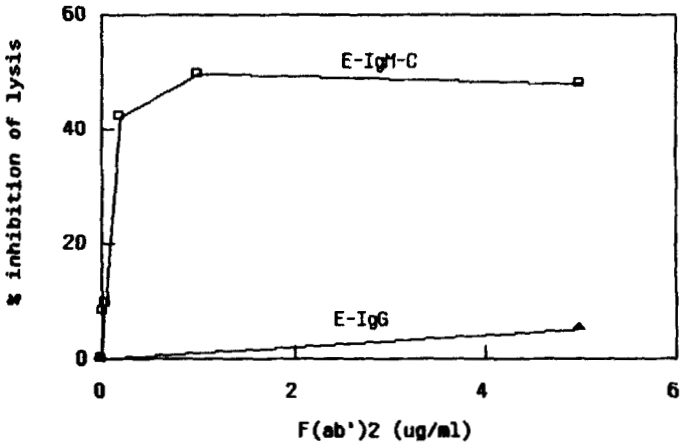

Figure 3. Effect of various concentrations of F(ab')$_2$ fragments of M1/70 on lysis of E-IgM-C (□) and E-IgG (▲) by TG-PM in an 18-hr ^{51}Cr-release assay with an E:T ratio of 1:2. Each point represents the mean of three experiments. Lysis of E-IgM-C and E-IgG in the absence of added F(ab')$_2$ was 42% and 41%, respectively.

PM (data not shown).

Kinetics of internalization and lysis of E. The kinetics were studied of phagocytosis and lysis by TG-PM of E, E-IgM-C, and E-IgM-C in the presence of M1/70 MAb. It was found that TG-PM phagocytosed the maximum amount of E-IgM-C in 1 hr, and of untreated E in 24 hr (Fig. 4*A*). Addition of M1/70 MAb to the assay delayed maximal uptake of E-IgM-C to 3 hr. ^{51}Cr was released from E-IgM-C 5 hr after introduction to the TG-PM, and maximum release was seen at 12 hr (Fig. 4*B*). Interestingly, M1/70 inhibited ^{51}Cr release (Fig. 4*B*) much more markedly than it inhibited phagocytosis (Fig. 4*A*). Although M1/70 had no effect on the magnitude of phagocytosis and only slightly inhibited its rate, ^{51}Cr release was markedly inhibited in both rate and extent.

Because the ^{51}Cr released from E-IgM-C into the medium roughly parallels the drop in internalized ^{51}Cr from its maximum at 1 hr, these data suggest that lysis of E-IgM-C by TG-PM is preceded by internalization of these sensitized E and lysis probably occurs intracellularly. Efforts to confirm this were difficult because phagocytic inhibitors such as dihydrocytochalasin B inhibited not only phagocytosis and lysis, but also inhibited rosette formation of E-IgM-C to TG-PM while having no effect on the integrity of the effector cells as assayed by trypan blue exclusion (data not shown). On the other hand, because E-IgM-C assayed in the presence of M1/70 are phagocytosed to the same degree as E-IgM-C assayed in the absence of MAb but at a slightly slower rate, and because M1/70 inhibits lysis by greater than 50%, these data suggest that internalization of E-IgM-C does not necessarily lead to its degradation within the 24 hr of this assay. The evidence for the intracellular nature of lysis was further supported by the inability of resident-PM to lyse E-IgM-C (data not shown). These cells have been previously shown to be similar to TG-PM in expression of the CR3 as measured by rosetting and anti-Mac-1 MAb binding (10, 20), yet are unable to mediate CR3-dependent phagocytosis (10).

Effect of longer incubations on lysis of unsensitized E. Assays lasting as long as 48 hr were performed to determine whether lysis of targets was solely a function of time after internalization or whether internalization by TG-PM via the CR3 specifically facilitated lysis of the

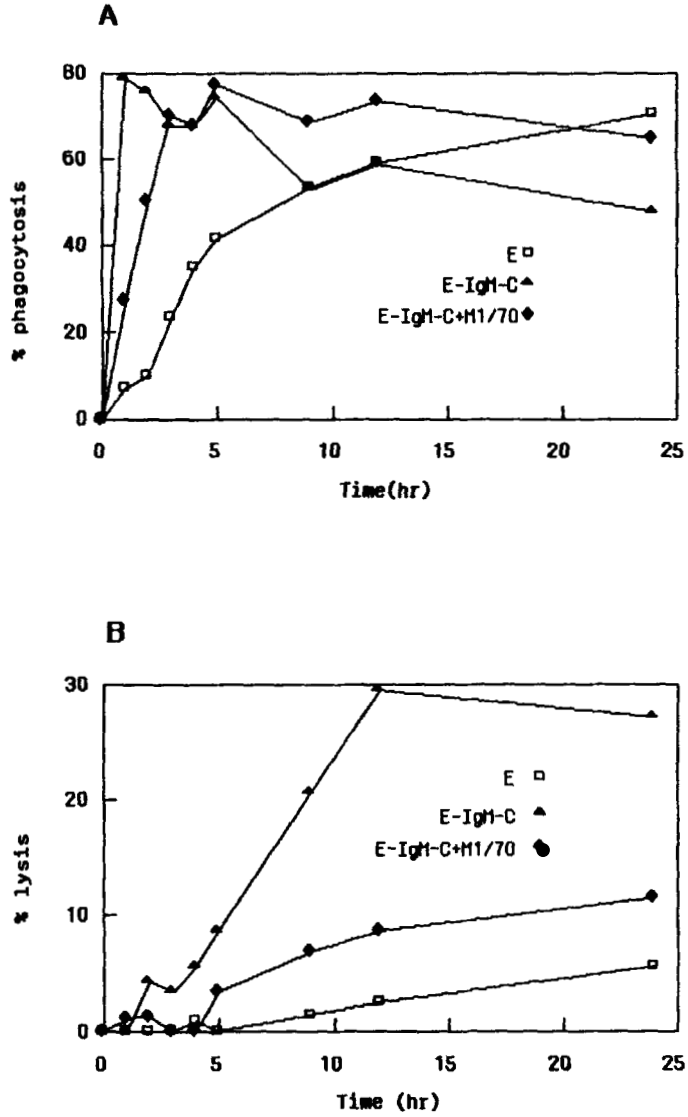

Figure 4. Kinetics of phagocytosis and lysis. Phagocytosis (*A*) and lysis (*B*) of E (□), E-IgM-C (▲), and E-IgM-C + M1/70 (◆) by TG-PM in a ^{51}Cr-release assay with an E:T of 1:2 were measured at 1, 2, 3, 4, 5, 9, 12, and 24 hr.

TABLE II

Effect of longer assay time on phagocytosis and lysis of E and E-IgM-C by TG-PM

Time of Assay	Percent Specific ^{51}Cr[a]			
	E		E-IgM-C	
	Released	Internal	Released	Internal
3 hr	3.2	5.7	1.1	42.0
24 hr	10.3	46.0	50.6	30.6
48 hr	28.9	31.4	60.2	17.9

[a] E:T of 1:2.

cells once they were internalized. As expected, maximum ingestion of E-IgM-C occurred within the first time point in the assay (3 hr), whereas a maximum and comparable number of unsensitized targets were ingested at 24 hr (Table II). However, the magnitude of ^{51}Cr released from the targets was much greater for E-IgM-C than for the unsensitized E approximately 24 hr after achieving maximum ingestion (24 hr after assay began for E-IgM-C and 48 hr for unsensitized E). These data suggest that the lysis of E-IgM-C is due not only to enhanced phagocytosis but also to a more active degradation mechanism once the E are ingested.

TABLE III

Selective lysis of E-IgM-C after internalization of E[a]

Target Added at 0 hr[b]	Target Added at 24 hr[c]	Percent Phagocytosis from 0 to 24 hr	Percent Lysis from 24 to 48 hr[d]
^{51}Cr-E	Cold-E-IgM-C	21.0	6.5
Cold-E	^{51}Cr-E-IgM-C	—	19.5
^{51}Cr-E-IgM-C	Cold-E	15.7	13.2
Cold-E-IgM-C	^{51}Cr-E	—	7.6

[a] This table is representative of two experiments. All points were done in triplicate.

[b] ^{51}Cr or cold targets (2×10^6) were added to TG-PM in parallel experiments. Wells containing ^{51}Cr-labeled targets were tested for percent specific phagocytosis (in table) and percent specific lysis, which were 17.4 and 42.7 for E and E-IgM-C, respectively, after 24 hr.

[c] ^{51}Cr or cold targets (2×10^5) were added to assays 24 hr after 0 hr targets were added.

[d] Lysis of ^{51}Cr-labeled targets was measured directly when labeled targets were added at 24 hr. When ^{51}Cr-labeled targets were added at 0 hr, percent of lysis in wells after 24 hr was subtracted from lysis in parallel wells incubated for 48 hr to calculate release in the 24- to 48-hr period.

These findings were confirmed when, in parallel experiments, TG-PM were incubated with ^{51}Cr-E or unlabeled E for 24 hr, so that a high degree of phagocytosis would occur; then unlabeled E-IgM-C or ^{51}Cr-E-IgM-C were added, respectively, to assays for an additional 24 hr and ^{51}Cr-release was measured (Table III). When the unopsonized E were labeled with ^{51}Cr, 21% of the E were internalized by 24 hr, and only 6.5% of the internalized ^{51}Cr was released into the supernatant during the second 24 hr. On the other hand, when the E-IgM-C were ^{51}Cr-labeled under the same assay conditions (i.e., they were added at 24 hr, which was after the TG-PM had already phagocytosed the E), there was 20% lysis of the E-IgM-C, which was greater than the magnitude of ^{51}Cr-released from already internalized E. These data support the finding that C3 not only enhances phagocytosis of targets, but also preferentially promotes the degradation of these opsonized targets. Furthermore, these data extend this finding by suggesting that internalization of E-IgM-C via the CR3 promotes a localized degradative event affecting that target but does not cause generalized lysis of internalized targets that were not phagocytosed via the CR3.

DISCUSSION

We have shown that TG-PM lyse E-IgM-C in an overnight assay. Data suggest that the interaction between iC3b on the target and the CR3 on the TG-PM is mandatory for effecting this lysis, because MAb that have been shown to inhibit rosetting of E-IgM-C to TG-PM via the CR3 (2) specifically inhibit lysis of E-IgM-C. A control MAb that binds to the β-chain of the CR3 but does not inhibit rosetting of E-IgM-C to TG-PM (19) similarly has no effect on the lysis of E-IgM-C. It appears that the lysis of the E-IgM-C is dependent on phagocytosis, as a period of rapid phagocytic activity by TG-PM precedes the gradual release of ^{51}Cr from E-IgM-C. It is likely that the internalized E-IgM-C are those that are lysed, for the increase in ^{51}Cr released into the assay supernatants roughly parallels the decrease of internalized ^{51}Cr from TG-PM incubated with E-IgM-C. In contrast to antibody-dependent killing mediated by the FcR (21), we found no evidence for lysis of extracellular target cells mediated by the CR3.

There have been some reports showing that the presence of C3 is able to augment the lytic activity of granulocytes and monocytes or macrophages towards IgG sensitized targets (11–15). It seems unlikely that there was any IgG present in our assay, because we used a monoclonal IgM anti-E antibody to fix the C and A/J serum, which was absorbed two times with equal volumes of packed E as a C source. Furthermore, TG-PM do not mediate lysis or phagocytosis of E-IgM or of E incubated with the absorbed A/J serum alone. Thus, we conclude that the lysis of E-IgM-C observed in our assay system is due solely to the interaction of E-IgM-C with the TG-PM and is independent of IgG. This finding is not surprising in light of the evidence showing that macrophages and monocytes can phagocytose C-coated particles under certain conditions, e.g., after exposure of monocytes or macrophages to phorbol esters (8), fibronectin-coated plates (7), lymphokines (9), or after elicitation with thioglycollate (10). In agreement with our findings, a recent report demonstrated no lysis of E-IgM-C by mouse TG-PM in a 2-hr assay (22), but the kinetics of phagocytosis and lysis at later time points were not investigated.

We found that there is a slow and steady internalization of unopsonized E by TG-PM. It is of interest that lysis of these unopsonized cells, once internalization has occurred, is much slower than of E-IgM-C. In fact, we found that TG-PM will preferentially lyse E-IgM-C even after they have already internalized unopsonized E. This was illustrated in experiments in which TG-PM were incubated with unopsonized E for 24 hr before the addition of E-IgM-C. Under these conditions, there was more lysis of the E-IgM-C than there was of the previously internalized E. These data then suggest that internalization of the E via the CR3 selectively compartmentalizes the E-IgM-C into a phagosome that becomes activated for lysis, whereas internalization of unopsonized E less efficiently activates the lytic mechanism.

Further support for this compartmentalization was seen in experiments in which TG-PM were exposed to E-IgM-C in the presence of anti-CR3 antibodies. Although M1/70 retarded the rate of phagocytosis of E-IgM-C by only several hours, the magnitude of lysis of the phagocytosed E-IgM-C was reduced by over 50% when M1/70 was present. It is possible that the E-IgM-C are being phagocytosed through the CR1 in this case due to cross-reactive specificities of the CR1 for iC3b and C3b (23), and that this type of phagocytosis is slightly slower than phagocytosis via the CR3 and does not trigger as strong a degradative response.

The less efficient lysis of E-IgM-C internalized in the presence of anti-CR3 suggests that the CR3, but not C per se, is important in promoting intracellular degradation. A trivial mechanism to explain the above data is that phagocytosis by TG-PM triggers a short-lived lytic burst that occurs very soon after the phagocytic event has started. Thus, many E-IgM-C, because they have been phagocytosed very rapidly, will be exposed to the lytic mechanism of the TG-PM, whereas unopsonized E or E-IgM-C assayed in the presence of M1/70 will not be exposed to the lytic event because of their slower phagocytosis. This mechanism has been ruled out, however, by experiments showing that E-IgM-C will be preferentially lysed even when they are introduced to the assay 24 hr after E. The mechanism by which CR3 bound to phagocytosed particles facilitates their lysis is an interesting area for further research.

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