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Enhanced degradation of soluble immune complexes by guinea-pig peritoneal macrophages in the presence of complement

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Summary. The role of complement in the processing of soluble immune complexes by guinea-pig peritoneal macrophages was studied in an homologous system *in vitro* by using immune complexes prepared with bovine thyroglobulin as the antigen and guinea-pig IgG2 antibodies. The simplest complexes showing complement activation and which were degradable by macrophages had a composition of Ag_1 Ab_{2-3}. Complement was shown to have an enhancing effect on the degradation of complexes which had an antibody: antigen ratio in the complexes which was at least 4 (Ag_1 Ab_4). The effect of size on complement activation and degradation of the complexes by macrophages was studied by employing the observation that immune complexes increase in size during their preparation. In the presence of serum as a complement source it was shown that degradation of small complexes by macrophages was inhibited whereas the degradation of large complexes was enhanced. The enhanced degradation of complexes in the presence of fresh serum did not occur in C4-deficient serum nor in EDTA-serum, which indicates that the observed effect is complement mediated. The experiments described here thus extend and confirm earlier studies using heat aggregated immunoglobulins and show that complement may play an important role in the elimination of immune complexes *in vivo*.

Abbreviations: $A(IgG_2)_n$, aggregated IgG_2, n is the number of IgG_2 molecules per aggregate; BTg, bovine thyroglobulin.

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INTRODUCTION

Macrophages play an important role in the clearance of circulating immune complexes and may thus prevent immune complex deposition in vessel walls (Mannik, Haakenstad & Arend, 1974). The mechanism by which macrophages eliminate immune complexes has mainly been studied *in vitro*. These studies have shown that macrophages can bind, internalize and degrade immune complexes or immunoglobulin aggregates via their Fc receptors alone (Leslie & Alexander, 1979; Knutson, Kijlstra & van Es, 1979b). Recent investigations have shown that complement can markedly enhance the elimination of immune complexes (van Snick & Masson, 1978) and immunoglobulin aggregates by macrophages, an effect which was shown to be dependent upon the ability of the aggregates to bind and activate complement components and which furthermore requires the presence of intact complement receptors (Kijlstra, van Es & Daha, 1979a; Kijlstra, van Es & Daha, 1979c). Although heat-aggregated immunoglobulins are a convenient model for

immune complexes (Knutson, Kijlstra, Lentz & van Es, 1979a), the introduction of artefacts due to heating cannot be excluded. The role of complement in the processing of immune complexes by guinea-pig macrophages *in vitro* was therefore investigated, using preformed soluble complexes of bovine thyroglobulin (BTg) and guinea-pig IgG2 anti-BTg antibodies.

The results presented in this paper show that both the capacity of the complexes to activate the complement system as well as their susceptibility to degradation by macrophages is dependent upon their size and composition.

MATERIALS AND METHODS

Immunoglobulin aggregates and immune complexes

Stable aggregates of heat-treated guinea-pig IgG2 were prepared as described earlier (Kijlstra *et al.*, 1979c). The aggregates used in this study contained approximately fifty IgG2 molecules per aggregate [A(IgG2)50]. Immune complexes were formed using BTg as the antigen and immunospecific IgG-anti-BTg as the antibody. Antibodies against BTg were raised in guinea-pigs by intramuscular immunization in Freund's complete adjuvant.

Antisera were adsorbed to a BTg immunoadsorbant and after extensive washing, the anti-BTg antibodies were eluted with 3 M LiBr. IgG2-anti-BTg was subsequently isolated by anion exchange chromatography (Leslie & Cohen, 1970) and radiolabelled with ^{131}I using lactoperoxidase (Thorell & Larsson, 1974). In large antigen excess 55% of the purified antibody was able to react with antigen, as measured by analysis of sucrose gradient profiles of immune complexes after ultracentrifugation for 16 hr at 210,000 *g* on 10%–30% w/v sucrose gradients. BTg was isolated as described earlier (Kijlstra, Knutson, van der Lelij & van Es, 1977) and iodinated with ^{125}I to a specific activity of approximately 0·5 mCi/mg. Soluble immune complexes were prepared by incubating a constant amount of 0·5 μg ^{125}I-BTg/ml with various amounts of immunospecific IgG2-anti-BTg for various time periods up to 2½ hr at 4° or at 37°. Hanks's medium containing 0·5% BSA (pH 7·4) was used as the diluent. Higher concentrations of BTg or longer periods of incubation resulted in the formation of insoluble immune complexes which precipitated after centrifugation for 10 min at 1500 *g* at room temperature. Analysis of immune complex size and composition by ultracentrifugation on sucrose gradients was performed as described earlier (Kijlstra *et al.*, 1977).

Complement activation

The ability of immune complexes to consume total complement activity was determined by incubating 100 μl immune complex samples with 100 μl of 1/500 diluted normal guinea-pig serum. After an incubation of 30 min at 37° the residual hemolytic CH50 activity was measured by adding 100 μl EA's and incubating for 60 min at 37°. The results were compared with reaction mixtures incubated with buffer, antigen alone, or antibody alone and expressed as percentage consumption.

The capacity of complexes to consume C3 activity was measured by incubating 100 μl immune complex samples with 100 μl C3 deficient guinea-pig serum supplemented with a 1/2000 final normal guinea-pig serum dilution so as to obtain 1·5 units of haemolytic C3 activity in the control samples. After an incubation for 30 min at 37°, residual C3 activity was measured by adding 100 μl EA's and incubating for 60 min at 37°. Half isotonic Veronal buffered saline, pH 7·5 containing 0·1% gelatin, 0·5 mM magnesium (Mg^{++}), 0·15 mM calcium (Ca^{++}) and 2·5% dextrose ($DGVB^{++}$) was used as the diluent.

Complement reagents

Normal and C4-deficient guinea-pig sera were obtained by heart puncture of ether anaesthetized guinea-pigs or C4-deficient guinea-pigs (kindly provided by Dr P. Lachman, Cambridge) and subsequently bred in our own laboratory. A C3-deficient serum was prepared by incubating normal guinea-pig serum with Cobra Venom factor bound covalently to Agarose A-5m (BioRad Laboratories, U.S.A.). Guinea-pig C1 (Kijlstra, van Es & Daha, 1979b) and guinea-pig C4 (Kijlstra *et al.*, 1979a) were isolated as published previously.

Immune complex degradation by macrophages

Non-stimulated guinea-pig peritoneal macrophages were isolated and allowed to adhere to lightly siliconized glass tubes as described elsewhere (Kijlstra *et al.*, 1979a). Degradation of immune complexes by macrophages was performed according to the TCA precipitation assay described earlier using immunoglobulin aggregates (Kijlstra *et al.*, 1979a; Kijlstra *et al.*, 1979c).

RESULTS

Effect of antibody: antigen combining ratio

The role of the antibody: antigen combining ratio on immune complex size, complement consumption and

degradation of immune complexes was studied using bovine thyroglobulin (BTg) as the antigen and guinea-pig IgG2-anti-BTg as the antibody. A constant amount of BTg (0·5 μg/ml) was incubated with varying amounts of IgG2-anti-BTg (0–6 μg/ml) for 30 min at 37° followed by a 2 hr incubation at 0°. Analysis of these complexes by sucrose gradient (10%–30%) ultracentrifugation (6 hr, 210,000 *g*) using ^{125}I-BTg and ^{131}I-IgG2-anti-BTg showed that an increase in the antibody:antigen combining ratio resulted in an increase in immune complex size and also in an increase in the molecular Ab:Ag ratio of the formed complexes (Table 1).

Complexes thus prepared were used to study the effect of the Ab:Ag combining ratio on the ability of the complexes to activate the complement system. The complexes were incubated with diluted guinea-pig serum reagents for 30 min at 37° after which residual CH50 or C3 activity was measured.

Appreciable CH50 and C3 consumption (Fig. 1) was observed starting at antibody concentrations of 1·5 μg antibody per ml. This mixture of complexes prepared at an Ab:Ag combining ratio of 7·6 contained mainly Ag_1 complexes with an Ab:Ag ratio of 2·4 (Table 1). A further increase in the Ab:Ag combining ratio resulted in a higher complement activation. The mixture prepared at the lowest combining ratio, which contained complexes with an Ab:Ag ratio of 1·8 showed very little complement activation.

The degradation of the various complexes described above by peritoneal macrophages in the presence or absence of fresh serum was studied by adding 100 μl

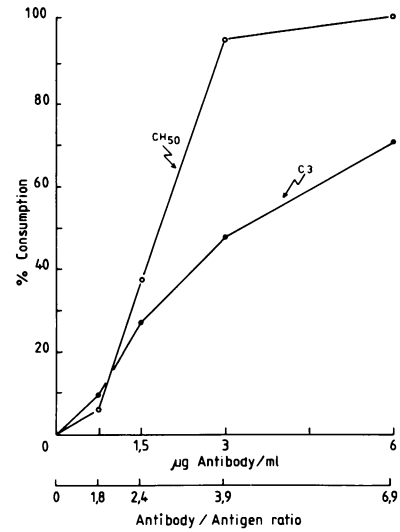

Figure 1. Complement activation by BTG anti-BTG immune complexes of various composition.

samples containing immune complexes with a different composition, together with fresh or heated serum. Degradation of the antigen was measured after a 2 hr incubation at 37°.

The results (Fig. 2) show that in the presence of heated serum a sharp increase in degradation occurs between final antibody concentrations of 0·75–1·5 μg/ml; a further increase resulted in a less marked increase in degradation. In the presence of fresh serum

Table 1. Molecular composition of BTg anti-BTg complexes prepared at various molar antibody:antigen combining ratios*

Ab μg/ml	Molar Ab:Ag† combining ratio	% Antigen in‡ Ag_1 complexes	% Antigen in‡ Ag_2 complexes	% Antigen in‡ > Ag_3 complexes	Ab:Ag ratio of complexes
0·75	3·8	88	6	6	1·8
1·5	7·6	78	14	8	2·4
3·0	15·2	62	24	14	3·9
6·0	30·4	31	26	43	6·9

* 0·5 μg of ^{125}I-BTg was mixed with varying amounts of ^{131}I-antibody and incubated for 30 min at 37° and 2 hr at 0°.

† Ab:Ag combining ratio was corrected because 55% of the antibodies were directed against BTg.

‡ After sucrose gradient centrifugation the percentage of antigen in Ag_1 and Ag_2 immune complex peaks was determined. The remaining antigen was present in complexes containing three or more antigen molecules.

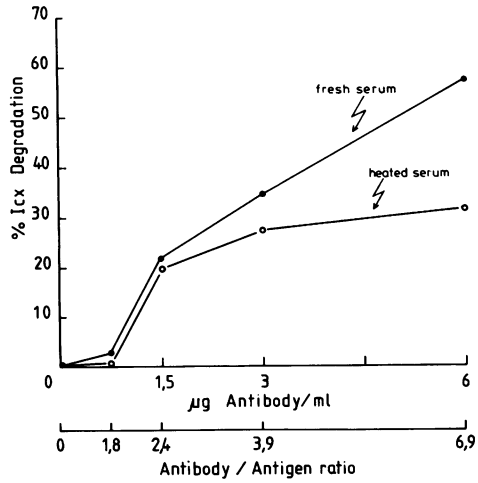

Figure 2. Degradation by macrophages of BTG anti-BTG complexes of various composition in the presence of 1% fresh (●——●) or 1% heated (o——o) serum. Complexes were incubated with macrophages during 2 hr at 37°.

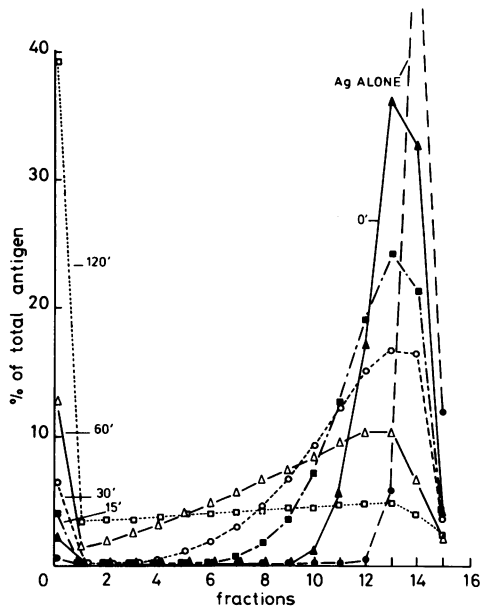

Figure 3. Sucrose gradient profile of immune complexes (0·5 μg BTG + 6 μg anti-BTG-IgG2/ml) formed at different incubation times at 37°. The bottom of the gradient is to the left (2 hr, 210,000 *g*).

as a complement source, enhanced degradation is only seen at the antibody concentrations of 3·0 and 6·0 μg/ml.

Effect of immune complex size

Since the preliminary experiments indicated that immune complex size was greatly influenced by the time and temperature of incubation, this observation was used in subsequent experiments to prepare complexes at a fixed antibody to antigen ratio, but varying the incubation at 37°.

A combining ratio of 0·5 μg BTg and 6 μg IgG2-BTg/ml was chosen because the previous experiments had shown that these complexes were capable of activating the complement system efficiently and furthermore because their degradation by macrophages was enhanced in the presence of complement. The effect of incubation time on immune complex size is shown in Fig. 3. Antigen and antibody were mixed together and incubated from 0 to 120 min at 37° after which 300 μl was layered on a sucrose gradient. Centrifugation (2 hr, 210,000 *g*) was immediately started and gradients were divided into fifteen fractions. As a control the sedimentation rate of antigen alone was also determined. It can be seen that when complexes were not pre-incubated at 37° only small complexes were formed which, however, still sedimented faster than antigen alone. At incubation times between 15 and 60 min the size of the complexes gradually increased as can be seen from the shift of radioactivity sedimenting deeper into the gradient. Incubation for 2 hr at 37° resulted in the formation of very large complexes of which 40% sedimented in the pellet of the gradient. These pelleted complexes were, however, still soluble by the criterion that they did not sediment by centrifugation for 10 min at 1500 *g* at room temperature. The effect of size of complexes on the ability to activate the complement system was measured by determining total haemolytic complement consumption by complexes which had been incubated for different time periods at 37° (Fig. 4). Although the small complexes (incubated for 0 min) were already capable of activating the complement system, an increase was seen in both CH50 and C3 consumption when the complexes had been allowed to become larger. Most CH50 units present in the incubation mixture were already consumed by complexes prepared for 30 min. The fact that C3 consumption levelled off for complexes prepared for 30–120 min, although the size of the complexes markedly increased during this time interval was an unexpected finding for which no satisfactory explanation has been found. To investigate the effect of com-

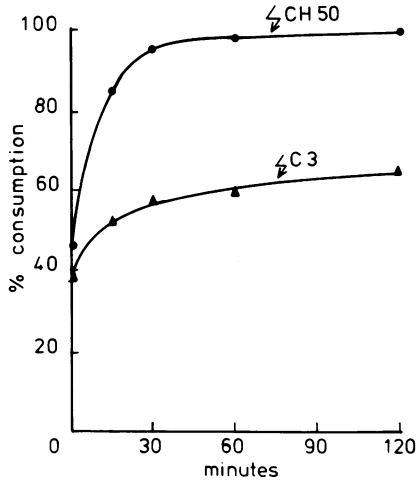

Figure 4. Effect of incubation time of immune complexes (0·5 μg BTG+6 μg anti-BTG-IgG2/ml) upon their ability to activate the complement system.

plement on the size of the complexes, the following experiment was performed.

Antigen (0·5 μg/ml) and antibody (6 μg/ml) were incubated for 0, 30 and 120 min at 37° after which one volume of medium or 5% fresh serum was added. After a subsequent incubation of 15 min at 37° the size of the complexes was analysed by sucrose gradient ultracentrifugation (1 hr, 210,000 *g*). Incubation of the different sized complexes with serum resulted in an increase in size for the three types of complexes tested (Fig. 5). The shift in size was, however, most pronounced for the complexes which were initially the largest. The amount of complexes localizing in the pellet (mol. wt $> 100 \times 10^6$) increased from 24% to 71% for the largest complexes (120 min incubation), whereas approximately 20% of the smallest complexes (0 min) had shifted deeper into the gradient. The effect of immune complex size on the degradation of complexes by macrophages was studied by allowing the complexes to grow in size by simply varying the incubation time and also by the addition of fresh serum to the incubation mixtures.

Antigen and antibody were incubated for different time intervals at 37° after which 1 vol. of ice cold medium or 5% normal guinea-pig serum was added. Immediately afterwards 100 μl samples were applied to macrophages and degradation of the complexes was determined after 1 hr incubation at 37°. As is shown in Fig. 6, degradation of the complexes in medium alone increased from 23% for the smallest complexes (0 min

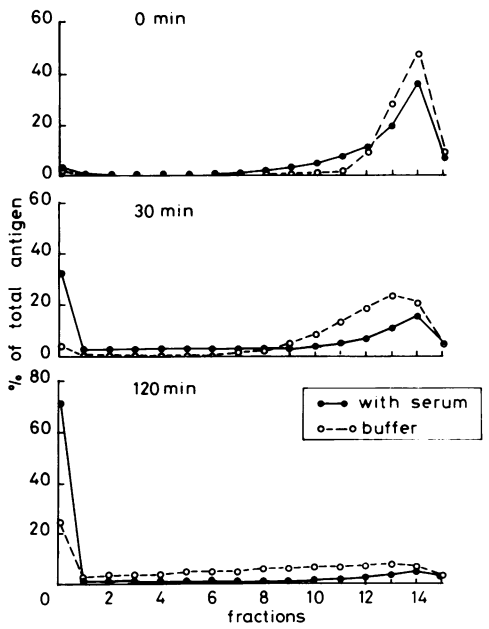

Figure 5. Effect of the addition of serum on the size of immune complexes pre-incubated for various time periods at 37°. The bottom of the sucrose gradient is to the left (1 hr, 210,000 *g*).

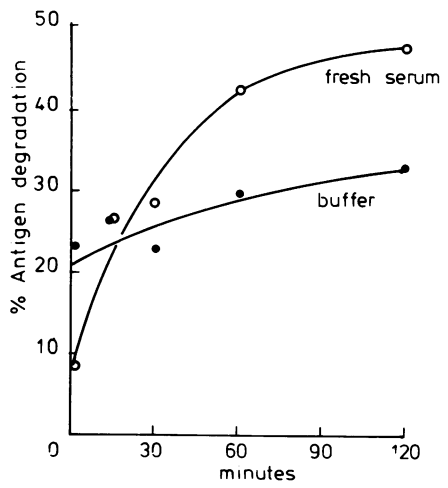

Figure 6. Degradation of immune complexes formed at various time (0–120 min) periods by macrophages in the presence of medium alone or in the presence of 2·5% fresh serum. Complexes were incubated with the macrophages during 60 min at 37°.

incubation) to 33% for the largest complexes (120 min incubation). Addition of fresh serum resulted in an inhibition of the degradation of the small complexes, whereas the degradation of larger complexes pre-incubated for 60 or 120 min was clearly enhanced compared to that seen in medium alone.

Effect of complement on immune complex degradation by macrophages

Earlier studies dealing with the effect of complement on the degradation of heat aggregated IgG2 by peritoneal macrophages had shown (Kijlstra *et al.*, 1979a) that enhanced degradation was dependent on the presence of an intact classical pathway up to at least C3. In the present study, using soluble BTg anti-BTg complexes, the requirement of an intact classical complement system for the stimulated degradation by macrophages was investigated with C4-deficient and EDTA serum.

Table 2 shows the results of an experiment whereby macrophages were offered BTG anti-BTg immune complexes (25 ng BTg, 300 ng IgG2 in 100 μl, 2 hr pre-incubated) in the presence of medium, normal serum, C4-deficient serum and C4-deficient serum to which isolated guinea-pig C4 or C1 had been added. Stimulated degradation was found with normal serum whereas degradation in C4-deficient serum was not significantly ($P > 0.01$) different from that observed in medium alone. Reconstitution of C4-deficient serum with isolated C4 increased the degradation significantly compared with controls but did not restore the degradation to values observed with normal serum. Dose–response studies with isolated and haemolytically active C4 did not result in an enhanced degradation exceeding the values shown in Table 2.

Table 2. Effect of C4-deficient serum on the degradation of immune complexes

Immune complexes in	% Degradation
Medium	23·6 ± 4·2
Normal serum	44·8 ± 3·0
C4-deficient serum	23·7 ± 2·0
C4-deficient serum + C4	29·5 ± 3·5
C4-deficient serum + C4 + C1	29·7 ± 3·2
C4-deficient serum + C1	32·1 ± 2·5
Medium + C4	24·0 ± 2·6
Medium + C1	32·1 ± 3·0

As earlier experiments had shown that the C4-deficient guinea-pig serum also had decreased C1 (25% of normal serum) and also decreased C1 inhibitor function (65% of control) the effect of C1 addition was also investigated during the same experiment (Table 2). The increase in degradation found by the addition of C4 and C1 to C4-deficient serum was, however, also found by the addition of C1 to medium or C4-deficient serum alone. Controls in which C4 was added to medium alone had no effect on the degradation of the complexes. These experiments thus indicate that activation of the complement system up to C4 causes a stimulated degradation, but that stimulation can also be observed by C1 alone.

Additional evidence for complement activation as a factor in the handling of immune complexes by macrophages was obtained by using EDTA serum. Macrophages were incubated with either immunoglobulin aggregates or immune complexes in medium alone, in medium containing 2·5 mM EDTA, in 2·5% normal serum and in 2·5% normal serum containing 2·5 mM EDTA.

As can be seen in Table 3, normal serum enhanced

Table 3. Effect of EDTA on serum-mediated increase of immune complex and immune aggregate degradation by macrophages*

Incubation medium	% Immune complex degradation	% AIgG2 degradation
Medium alone	14·0 ± 1·1	18·3 ± 2·4
Medium + 2·5 mM EDTA	10·3 ± 2·2	18·7 ± 4·4
2·5% serum	35·3 ± 2·8	45·6 ± 1·5
2·5% serum + 2·5 mM EDTA	6·9 ± 1·2	10·5 ± 3·8

* Immune complexes (25 ng BTg in 100 μl) or immune aggregates (50 ng in 100 μl) were incubated for 60 min at 37° with peritoneal macrophages after which the % of degradation was determined.

the degradation of both aggregates and complexes. This stimulation was blocked by adding 2·5 mM EDTA whereas EDTA alone had no effect on the degradation. This latter experiment thus suggests that C activation is necessary for the stimulating effect of serum on the degradation of immune complexes and aggregates by macrophages.

DISCUSSION

The results described in this paper show that both the antibody:antigen ratio as well as the size of the immune complexes are important factors defining the biological properties of the complex. Both complement activation as well as the susceptibility to degradation by macrophages only occur using complexes prepared in a molecular antibody excess. The simplest complexes that were degradable by macrophages in the study presented here were complexes having a composition of Ag_1 Ab_{2-3} (Table 1, Fig. 2). Appreciable complement activation also started at this immune complex composition (Fig. 1). Earlier studies by Shinomiya & Koyama (1976) also showed an effect of the antibody to antigen combining ratio on the processing of complexes by macrophages in serum-free media. These authors, however, did not define the size nor composition of the administered complexes. In the study presented here, complement was shown to have an enhancing effect on the degradation of those soluble complexes which had a molecular formula of Ab_1 Ab_4. Similar results were claimed by others (van Snick & Masson, 1978), who used a heterogeneous population of 'soluble' immune complexes, whereby the solubility of the complexes was determined by visibility, whereas in our study solubility was defined as those complexes which were precipitable during centrifugation for 10 min at 1500 *g*. In their study (van Snick & Masson, 1978) the percentage of degradation was so low (less than 1% of the offered complexes), that a selective degradation of insoluble complexes present in the mixture can easily be envisioned.

In our hands, the incubation of soluble immune complexes with serum markedly enlarged the size of the immune complexes; an effect which could also be reproduced by the addition of isolated C1 or C1q (Kijlstra, unpublished observations). In this respect the immune complexes used here differ from IgG aggregates used earlier, IgG aggregates only slightly increased in size when incubated with low concentrations of serum and incubation of aggregates with isolated C1 resulted in a dissociation of the aggregates (Kijlstra *et al.*, 1979b, c).

Reconstitution of C4-deficient serum with purified haemolytically active C4 did not restore immune complex degradation to the values obtained with normal serum. Although this may be attributed to the presence of lower levels of C1 and C1 inhibitor in C4 deficient serum other unknown factors may also play a role. To further investigate the role of C in immune complex degradation we investigated the effect of EDTA. Dose–response studies showed that the minimal amount of EDTA needed to inhibit the serum-induced enhanced degradation was 2·5 mM. At this concentration EDTA had no significant effect on the degradation of aggregates or immune complexes by macrophages in medium alone. The inhibition observed in EDTA serum compared with EDTA medium could be due to inhibitory effects of monomeric IgG.

A further difference between immune complexes and IgG aggregates is the stability. Both the incubation temperature as well as the incubation time had a marked effect on the composition and therefore the handling of the complexes by macrophages. This was shown to be due to the continuous growing size of the soluble complexes. After an overnight incubation at 4°, approximately 30% of the antigen was precipitated when complexes in relatively large antibody excess were made (0·5 μg BTg + 6 μg IgG2 anti-BTg per ml). The enlargement of immune complexes upon incubation and also the dissociation of complexes upon dilution as reported earlier (Kijlstra *et al.*, 1977) are phenomena which complicate these experiments. On the other hand the increase in size can be used as a tool to study immune complexes of various sizes. The changes in size during the actual experiments were kept at a minimum by keeping the time needed for analysis by ultracentrifugation and the incubation times with the macrophages relatively short. To avoid precipitation of immune complexes most experimental workers have used complexes prepared in antigen excess. These complexes are, however, mostly not susceptible to degradation by macrophages and are less effective in their capacity, to activate the C system (G. Doekes, L. A. van Es, A. Kijlstra and M. R. Daha, manuscript in preparation).

Immunoglobulin aggregates stabilized in albumin have the advantage of staying constant in size, but have a disadvantage in that the effects of heating may influence the biological properties of the aggregates.

The enhancing effect of complement on the degra-

dation by macrophages, however, applies to both immune complexes and immunoglobulin aggregates although the manner in which the enhancing effect is reached may be different in the two cases. With the aggregates it may be that binding of C3 on the aggregates, without remarkable enlargement of the aggregate, increases its binding to the macrophage, whereas with complexes both the binding of C3 to the immune complexes as well as cross-linking by C1q may occur. Further studies employing inhibition techniques with free IgG and C3 will shed more light on this matter.

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