THE EFFECT OF COMPLEMENT ON THE INGESTION OF
SOLUBLE ANTIGEN-ANTIBODY COMPLEXES AND IgM
AGGREGATES BY MOUSE PERITONEAL MACROPHAGES*

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In vivo, the elimination of soluble antigen-antibody (AgAb) complexes by the
reticuloendothelial system is apparently independent of complement (1). This con-
trasts with the promoting effect of complement on the phagocytosis of Ab-coated
particles. In this case, C3 and IgG act in synergy. IgG, by reacting with Fc-receptors,
stimulates particle ingestion, but is relatively inefficient at inducing particle binding.
C3 mediates the binding of the particle to the phagocyte without direct stimulation
of the ingestion (2). The present work deals with the opsonic activity of complement
on soluble material, and reports the following two observations. First, in contrast to
what has been observed in vivo, complement markedly promotes the ingestion by
mouse peritoneal macrophages (MPM) of soluble AgAb complexes containing IgG
antibodies. However, with 125I-labeled complexes, this effect is evident only when the
release of degradation products is considered. Using 59Fe-labeled human transferrin
(Tf) as Ag in the complexes, it is possible to demonstrate directly the increase of
uptake induced by complement because, as it will be shown, 59Fe is not released from
the macrophages after digestion of its carrier. Second, contrasting with the results
obtained with IgM-coated particles (2), soluble heat-aggregated human IgM is
ingested in significant amounts in the presence of complement, indicating that the
endocytosis of soluble material, in contrast to particles, does not necessarily require
the involvement of Fc receptors.

Materials and Methods

Cells. Nonstimulated macrophages were collected from the peritoneal cavity of normal
female NMRI mice. In some experiments, 129/Sv mice maintained under specific pathogen-
free conditions were used. The cells were processed as previously described (3) with slight
modifications. Briefly, a 10-ml vol of basal medium of Eagle (BME) containing 20 U of heparin
per milliliter was injected into the peritoneal cavity, and reaspirated after abdominal kneading.
After centrifugation, the cells were resuspended in BME containing 20% heat-decomplemented
fetal calf serum (FCS), 50 U/ml penicillin, and 50 µg/ml streptomycin. About 3.107 cells were

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Abbreviations used in this paper: Ab, antibody; Ag, antigen; AT; mouse antiserum against human
transferrin; BME, basal medium of Eagle; E-IgM, erythrocytes coated with IgM, E-IgM-C, E-IgM and
complement; FCS, fetal calf serum; FMS, fresh mouse serum; HMS, mouse serum decomplemented by
heating; MPM, mouse peritoneal macrophages; PBS, phosphate-buffered saline; TCA, trichloroacetic
acid; Tf, human transferrin.
THE EFFECT OF COMPLEMENT ON SOLUBLE COMPLEXES

seeded per well in Sterilin tissue culture plates (Teddington, Middlesex, Great Britain). After about a 1-h incubation at 37°C in a 10% CO2 atmosphere, the adherent macrophages (5-7·105 cells per well) were washed free of other cells with BME, and reincubated with the appropriate medium.

Reagents.
Human iron-free Tf was from Behring Institute (Marburg/Lahn, West Germany). Antiserum against Tf (ATf) was obtained from NMRI mice after weekly subcutaneous injections of 100 µg of antigen suspended in complete Freund's adjuvant. For labeling Tf, the protein, dissolved in phosphate-buffered saline (PBS) containing 0.05 M NaHCO3, was mixed with [59Fe]citrate in sufficient amounts to saturate the protein up to 80% of its iron-binding capacity. Tf-ATf complexes were prepared in 3-, 5-, and 10-fold Ag excess by adding the appropriate amount of heat-decomplemented mouse antiserum to labeled Tf. After a 1-h incubation at 37°C, the complexes were left for 3 days at 4°C before use. Even after 3 wk, no precipitate was visible. The amount of Tf bound by the anti-Tf antibodies was measured for each preparation of immune complexes by density gradient ultracentrifugation (4). The distribution of complexes was as follows. For the preparation in threefold Ag excess, 13% of the complexes sedimented as a 13.5 S peak, 30% as a polydisperse fraction (13.5-30 S), and 57% were concentrated in the pellet (>30 S). For the preparation in 5- and 10-fold Ag excess, respectively, 27 and 34% were in the 13.5 S peak, 45 and 50% in the polydisperse fraction, and 30 and 16% in the pellet.

A human IgM monoclonal component was kindly provided by Dr. J. P. Vaerman (Université Catholique de Louvain, Brussels, Belgium), and aggregated by heating at 63°C for 30 min. After such treatment, the preparation was slightly opalescent, but no precipitate was visible. In gradient ultracentrifugation, all the aggregates were recovered in the pellet corresponding to material of >30 S. No IgG (<0.1% of the protein content) was detected in the IgM solution by immunonephelometry (AIP system, Technicon Instruments Corp., Tarrytown, N. Y.).

As a source of complement, we used fresh NMRI serum (FMS) or guinea pig serum. The sera were decomplemented by heating at 56°C for 30 min, by incubation with zymosan (20 mg boiled zymosan per milliliter of serum, 2 h at 37°C), or with hydrazine (5). Human IgM and human Tf were labeled with 125I by the chloramine T method.

Methods. To measure the amount of immune complexes or IgM aggregates associated with MPM, the radioactivity was counted on the cellular extracts obtained by 1% Triton X-100 (Robin & Haas Co., Philadelphia, Pa.), the value being corrected for the radioactive material in the absence of cells. Before extraction, MPM were washed once with 3 ml and twice with 1 ml PBS, and then counted under the inverted microscope.

The degradation products released by the cells into the medium were recovered in the supernate of the culture medium after precipitation of proteins by 10% trichloroacetic acid (TCA). The TCA-soluble radioactivity was corrected by subtracting the value obtained in controls consisting of the supernates of the culture medium precipitated by TCA after having been incubated with the radioactive material in the absence of cells.

To check whether MPM were activated or not, experiments with sheep-erythrocytes coated with IgM antibodies and complement (E-IgM-C) were done following a method similar to that used by Bianco et al. (6). A 10% suspension of sheep erythrocytes was incubated at 37°C for 30 min with a subagglutinating concentration of mouse IgM antibodies prepared as described hereafter. After two washings with PBS, the IgM-coated cells were resuspended in BME containing 20% FMS which had previously been absorbed with sheep erythrocytes, and incubated for 30 min at 37°C. The cells were washed again twice with PBS, and finally resuspended in BME before their addition to MPM. IgM antibodies against erythrocytes had been isolated from the mouse anti-sheep erythrocytes serum by gel filtration on Ultrogel AcA 34 (LKB, Bromma, Sweden) followed by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) for retaining the residual IgG molecules. Trace amounts of IgG2b antibodies were removed by passage on a column of insolubilized rabbit anti-IgG2b (7). Before counting the erythrocytes ingested by MPM, the latter were washed thrice with PBS, and the noningested erythrocytes lysed by a 45-s contact with hypotonic solution (fivefold diluted PBS). The monolayer was then fixed with 5% glutaraldehyde before examination by phase-contrast microscopy.

For the fluorescence study, the antigen of the immune complexes i.e., human Tf was labeled with fluorescein isothiocyanate. The MPM from 129/Sv mice were cultured in dishes 3001
from Falcon Plastics (Division of BioQuest, Oxnard, Calif.) for 1 h in the presence of the labeled complexes in threefold Ag excess, with fresh or heated mouse serum. After three washings with medium at 37°C, the cells were examined under a cover slip with an ultraviolet microscope.

Results

The Effect of Complement on the Uptake and Digestion of AgAb Complexes. MPM were incubated for various times with $^{125}$I-Tf-ATf complexes (5 μg Ab/ml) in BME containing 20% FMS or mouse serum decomplemented by heating (HMS). After a slight increase during the first 3 h, more evident with FMS, the radioactivity associated with the cells remained almost constant (Fig. 1, lower panel).

When the degradation products detected in the medium as TCA-soluble $^{125}$I were considered, their rate of appearance after incubation with FMS was about four times higher than after incubation with HMS (Fig. 1, upper panel, and Table I). Similar results were obtained with AgAb complexes which contained labeled Ab rather than labeled Ag (data not shown).

After 2.5 h, the radioactivity associated with $10^5$ MPM remained almost constant at a value representing 0.185% of the complexes per milliliter medium after incubation with complement, and 0.049% after incubation without complement. When expressed in percents of these values, the rates of release of degradation products were,

![Graph showing the effect of FMS on the uptake of $^{125}$I-labeled Tf-anti-Tf complexes by MPM (lower panel) and on the release of degradation products from $^{125}$I-Tf (upper panel). The results were expressed in percents of the AgAb complexes per milliliter of medium. Vertical bars represent 1 SD calculated from triplicate experiments.](image-url)
THE EFFECT OF COMPLEMENT ON SOLUBLE COMPLEXES

TABLE I

| Conditions of incubation | Rate of uptake* | Rate of release‡ |
|--------------------------|----------------|-----------------|
| With FMS                | 0.182 (SD = 0.009) | 0.228 (SD = 0.033) |
| With HMS                | 0.058 (SD = 0.007)  | 0.065 (SD = 0.016)  |

* \( \frac{\text{Fe associated with 105 MPM}}{\text{[Fe-Tf-ATf/ml medium]h}} \times 100 \)
‡ \( \frac{TCA \text{ soluble 125I released by 105 MPM}}{\text{[125I-Tf-ATf/ml medium]h}} \times 100 \)

respectively, 123 and 133%/h in the experiments done with and without complement. Hence, the digestion rates did not differ significantly, as confirmed by the study of the half-lives of complexes in MPM described later.

To see whether the effects of FMS could be due to an enhancement of the extracellular proteolysis of the complexes, MPM were incubated in BME containing 20% FMS or HMS. After 8 h, this medium was recovered and reincubated for 10 h at 37°C with 125I-Tf-ATf complexes in the absence of cells. Extracellular proteolysis was calculated from the difference of the TCA-soluble radioactive material recovered from this medium and from control medium not preincubated with cells. In 1 ml of medium corresponding to a culture of 105 MPM, only 0.15 and 0.16% of the Ab-associated Tf was digested by extracellular proteolysis in the presence of HMS or FMS, respectively.

The endocytosis experiments with 125I-Tf-ATf complexes (50 μg Ab/ml medium) were repeated with BME containing 20% guinea pig serum as a source of complement. The same results as with mouse serum were obtained. To ascertain that complement acted via C3 and not via C1, which could increase the size of the complexes, guinea pig serum was treated with zymosan or hydrazine which affect C3 without interfering with C1. Similar results were obtained with zymosan- or hydrazine-treated serum as with heated serum (Fig. 2).

The Effect of Complement on the Half-Life of AgAb Complexes in MPM. After a 2-h incubation with 125I-Tf-ATf complexes in the presence of FMS or HMS, MPM were washed and reincubated, in the absence of AgAb complexes, in BME containing 20% decomplemented FCS. After various times, the radioactivity remaining in the cells was measured (Fig. 3). No difference was observed in the decay of radioactivity, between the two incubation conditions, i.e., in the presence or absence of complement. In both cases, after 1 h, ≈20% of the initial radioactivity was released from the cells, a value differing markedly from that estimated earlier in the experiment with continuous feeding of MPM with complexes (120–130%/h). This difference could be due to handling of the cells. In the half-life experiment, the repeated washings at room temperature altered the morphology of MPM, which lost their characteristic spread pattern and acquired a round shape. Presumably, the metabolic activity of the cells was also modified, which could explain the decreased digestion and excretion rate of the ingested material.

Effect of Complement on the Ingestion of AgAb Complexes Containing Tf Labeled with 59Fe. MPM were incubated with 59Fe-Tf-ATf complexes in threefold Ag excess for 17 h with or without complement. After washing, the cells were reincubated, in the absence of AgAb complexes, in BME containing 20% decomplemented FCS. No
J. L. Van Snick and P. L. Masson

significant decay of the radioactivity of the cells was observed over a 26-h period, whether the incubation had been in the presence or absence of complement (Fig. 4). Hence, the radioactivity of the cells corresponded to the total amounts of AgAb complexes that had been picked up, and the effect of complement could be measured
The effect of complement on soluble complexes

Fig. 4. Persistence of 59Fe in MPM after ingestion of 59Fe-Tf-anti-Tf complexes in the presence or absence of complement. Vertical bars represent 1 SD calculated from triplicate experiments.

Fig. 5. Uptake of 59Fe-Tf-anti-Tf complexes by MPM in the presence of FMS or HMS. Note the point (■) corresponding to the uptake of 59Fe-Tf free of antibody. Vertical bars represent 1 SD calculated from triplicate experiments.

directly (Fig. 5). With complement, the rate of uptake was found to be about three times faster than without. The extrapolation of the uptake regression lines to zero time indicated that, with complement, 10^6 MPM instantaneously bound 0.3% of the complexes present in 1 ml medium and, without complement, <0.01%.

No significant difference (Table I) was found between the uptake rates of AgAb complexes calculated in the experiment with 59Fe-Tf-ATf complexes (Fig. 5) or from the release of degradation products of 125I-Tf-ATf complexes (Fig. 1).

When complexes in 5- or 10-fold Ag excess were used for the uptake experiments, the enhancing effect of complement was still clearly visible (Table II). However, with
such excess of $^{59}$Fe-Tf, it was found necessary to correct the results for the uptake of free Ag. This was done in control experiments performed with $^{59}$Fe-Tf alone.

To see whether the $^{59}$Fe-Tf-ATf complexes were ingested or just bound to the cell surface, MPM were treated with trypsin which is known to destroy C3-receptors (8). After various incubation times with the immune complexes, the cells were washed twice with 2 ml BME, and reincubated for 30 min at 37°C in BME with or without 0.5 mg/ml trypsin. Such treatment did not decrease significantly the radioactivity of the cells. As the trypsin treatment was repeated after different incubation times, it was possible to calculate the rate of uptake. This was 0.194% of the complexes in 1 ml medium/h (SD = 0.013), a value not significantly different from that obtained in the experiments done without trypsin (0.212%/h, SD = 0.015).

In control experiments, MPM were exposed first to trypsin for 30 min before incubation with the immune complexes. In such conditions the enhancing effect of complement was abolished. After a 30-min incubation with complexes, the radioactivity associated with the cells reached only 6% of the expected value. It must be noted that trypsin did not affect the $^{59}$Fe-Tf-ATf complexes, as shown by gradient ultracentrifugation.

The ingestion of the complexes was confirmed by fluorescence data. After incubation with immune complexes containing fluorescein-labeled Tf for 1 h, nonfixed MPM showed large cytoplasmic patches of fluorescein (Fig. 6, A). The intensity of fluorescence was markedly lower in MPM incubated with complexes in the absence of complement (Fig. 6, B).

**Effect of Complement on the Uptake of $^{125}$I-Labeled Aggregated Human IgM by MPM.** MPM were incubated with $^{125}$I-labeled heat-aggregated IgM (5 μg/ml) in the presence or absence of complement (20% fresh or heated guinea pig serum). As a source of complement, we used guinea pig serum rather than mouse serum as human IgM aggregates were apparently unable to activate mouse complement. The radioactivity of the cells increased faster in the presence of complement. Significant differences were already observed after 30 min. After 3 h, the radioactivity tended to become constant in the absence of complement (Fig. 7, lower panel).

The effect of complement was particularly evident when the release of degradation products was considered. Its rate was 22 times higher than after incubation with heated guinea pig serum (Fig. 7, upper panel).

To check whether aggregated IgM had been ingested and not digested at the cell surface, MPM having been exposed to $^{125}$I-IgM aggregates were treated with trypsin to destroy C3-receptor. MPM were incubated with 10 μg/ml $^{125}$I-IgM aggregates in the presence or absence of complement (20% fresh or heated guinea pig serum). After
THE EFFECT OF COMPLEMENT ON SOLUBLE COMPLEXES

Fig. 6. MPM after 1 h incubation with Tf-anti-Tf complexes in threefold Ag excess containing fluorescein-labeled Tf. (A) After incubation with fresh mouse serum. (B) After incubation with heated mouse serum. X 400.

Fig. 7. The effect of fresh guinea pig serum as a source of complement on the uptake by MPM of 125I-labeled heat-aggregated human IgM (125I-HA-IgM) (lower panel) and on the release of degradation products as TCA-soluble 125I (upper panel). Bars represent 1 SD calculated from triplicate experiments.

various times, the cells were washed with PBS, and then reincubated at 37°C for 30 min in BME with or without 0.5 mg/ml trypsin. The amount of radioactivity associated with the cells at various time intervals indicated that ≈50% of the material bound to the cells in the presence of complement was ingested, the rest being at the
J. L. van Snick and P. L. Masson

Fig. 8. Effect of trypsin treatment of MPM on the uptake of $^{125}$I-labeled heat-aggregated human IgM in the absence or presence of fresh guinea pig serum as a source of complement. After incubation for various times with $^{125}$I-IgM aggregates with or without complement, the cells were reincubated with or without trypsin for 30 min. After appropriate washings the radioactivity of these cells was measured. The uptake after incubation without complement but after trypsin treatment is indicated by O. Bars represent 1 SD calculated from triplicate experiments.

cell surface (Fig. 8). Trypsin released the IgM aggregates from the cell surface probably by digesting the IgM itself as well as the C3 receptors, as gradient ultracentrifugation of trypsin-treated $^{125}$I-IgM-aggregates showed complete digestion of the protein: all the radioactivity remained at the top of the gradient.

Controls of the Nonactivated State of MPM. To exclude the possibility that the cells used for the experiments were activated macrophages, the experiments with aggregated IgM were repeated on MPM collected from 129/Sv mice maintained in specific pathogen-free conditions. The results were similar to those obtained with conventional mice.

Further proof that these MPM were not activated was provided by the fact that E-IgM-C attached to macrophages but were not ingested. MPM from 129/Sv mice were incubated in BME with E-IgM or E-IgM-C prepared as described in Materials and Methods. After 1 h at 37°C, appropriate washing, and osmotic lysis of noningested erythrocytes, those ingested by MPM were counted by phase-contrast microscopy. The number of E-IgM-C attached to 100 MPM was 638 ($\pm$ 33), whereas the number of ingested E-IgM-C was 9 ($\pm$ 3). No ingested E-IgM was detected.

Discussion

The present work shows that complement enhances the ingestion of soluble AgAb complexes in various Ag excess by MPM. With $^{125}$I-labeled complexes, this activity became evident only when the release of degradation products was measured. When the complexes were labeled with $^{59}$Fe, using Tf as Ag, the uptake was measured directly as the metal was not excreted by the cells.

The fast elimination of degradation products by MPM after incubation of immune complexes with complement raised the question of a possible role of complement in
their intracellular digestion. By stimulating the digestion, complement could indirectly accelerate the ingestion. Comparing the half-lives of complexes after their uptake by MPM in the presence or absence of complement did not show any difference. This suggested that complement did not promote the digestion.

That this digestion occurred inside the macrophages was indirectly shown by the weak proteolytic activity of the medium having been in contact with MPM, and by the lack of effect of trypsin added after the cellular uptake of complexes. This observation suggests that the complexes were not anymore on the cell surface but were already ingested when the trypsin was added. However, it was possible that the C3-receptors became unaccessible to trypsin when bound to the complexes. So, we checked the ingestion of complexes by fluorescence. This confirmed that complement did promote the endocytosis.

Our results contrast with the observation of Mannik and Arend (1) who reported that, in vivo, the clearance of AgAb complexes was independent of complement. This discrepancy could be explained by the great difference in the proportion of complexes vs. phagocytes in the two experimental systems. On one hand, the amount of immune complexes injected by Mannik and Arend being much lower than the phagocytic capacity of the reticuloendothelial system of the animals, the complexes were cleared so fastly through Fc-receptors that the complement could not accelerate the process. On the other hand, in our in vitro experiments, the number of cells being restricted, the opsonic activity of complement became evident. The in vivo experiments consisted of a single injection of immune complexes, whereas in pathological conditions such as infections, Ag are released and immune complexes produced in a continuous way. In such a situation, the clearance of complexes is presumably facilitated by C3. One could then wonder why immune complexes that contain C3 persist in the blood of patients with various diseases (9, 10). We suppose that, in fact, these complexes are eliminated but continuously replaced because of the permanent supply of Ag.

Our second observation dealt with the stimulating activity of complement on the ingestion of IgM-aggregates. Ehlenberger and Nussenzweig (2) reported that complement enhanced the adherence of IgM-coated erythrocytes but not their ingestion. The endocytosis occurred only when the particles were attached to the Fc-receptor alone or to both Fc- and C3-receptors. In our work, complement did promote the attachment of IgM-aggregates, but also their ingestion.

Bianco et al. (6) have shown that macrophages activated by endotoxin or thioglycolate are capable of ingesting C3-coated particles without binding to Fc-receptors. So, our results could perhaps be explained by the activation of macrophages because of some latent infections of the animals. This was improbable as the cells did not show any morphological signs of activation and did not ingest erythrocytes coated with IgM antibodies and complement.

The differences between the fates of particulate and soluble complexes could be explained by the fact that ingestion of particles requires more important modifications of the phagocyte membrane than does the ingestion of soluble complexes. Experiments with cytochalasin indicate that ingestion via Fc-receptors requires the functional integrity of microfilaments, whereas C3-mediated endocytosis would be less dependent on motile structures (11). Hence, nonactivated macrophages could ingest C3-coated material, provided it is soluble.

We have shown elsewhere that optimal concentrations of rheumatoid factor pro-
mote, in the absence of complement, the endocytosis of IgG-containing complexes. In excess, it prevents their ingestion presumably by hiding the Fc regions of IgG (12). As IgM-rheumatoid factor is detected in large excess in many diseases with circulating AgAb complexes, it appears now likely that these complexes coated with IgM-rheumatoid factor can be ingested by macrophages through their C3-receptors.

Summary

Complement was found to stimulate markedly the ingestion of soluble antigen-antibody complexes by mouse peritoneal macrophages. This was shown indirectly by measuring the release of degradation products when the complexes were labeled with $^{125}$I, or directly when the antigen, that was human transferrin, was labeled with $^{59}$Fe. In this case, the metal which was released from human transferrin inside the cells was not excreted, and its accumulation in the macrophages was a direct index of the uptake of immune complexes. The decay of radioactivity in macrophages after ingestion of $^{125}$I-labeled complexes was similar when they were taken up with or without complement, indicating that complement acts primarily on ingestion and not on digestion or excretion. The ingestion of complexes was morphologically confirmed using fluorescein-labeled antigen in the immune complexes.

The opsonic effect of complement was also observed with IgM aggregates indicating that soluble complexes can be ingested through complement receptors without involvement of Fc-receptors, as required for particulate antigen-antibody complexes.

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