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Interaction of complement solubilized complexes with mouse peritoneal macrophages and their clearance and tissue uptake

N. TAKAHASHI, T. FUJITA, Y. TAKATA* & N. TAMURA *Department of Immunology, Insitute of Basic Medical Sciences, University of Tsukuba, Ibaraki, Japan*

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SUMMARY

To clarify the biological activity of complement solubilized immune complexes, we studied their interaction with mouse peritoneal macrophages. The solubilized complexes lost their binding affinity for C3b receptor and Fc receptor but still bound to MPM mainly via the C3bi receptor (CR3). When solubilized immune complexes were injected into mice, they were more rapidly removed from the circulation than antigen excess soluble complexes and taken up by the liver Kupffer cells. Therefore, the solubilized complexes could be catabolized by the reticuloendothelial system, mainly in the liver. Probably, CR3 plays an important role in this process.

Keywords complement solubilized complexes macrophage complement receptors Fc receptor

INTRODUCTION

Antigen–antibody aggregates can be solubilized by complement activation, yielding immune complexes of relatively small mol. wt which contain antigen, antibody and complement (Miller & Nussenzweig, 1975). During the solubilization process, the immune complexes lose various biological functions which are known to be associated with large latticed immune complexes. For instance immune complexes solubilized in fresh serum no longer activate complement. Binding activities of these complexes to mouse lymphocytes (Miller, Saluk & Nussenzweig, 1973) and C3b receptors (CR1) on human erythrocytes (Takahashi, Takahashi & Hirose, 1980) are abolished. However, it is not known whether solubilized complexes also lose their affinity for macrophages in the reticuloendothelial system (RES). In this report, we analyzed the interaction of solubilized complexes with mouse peritoneal macrophages and studied the clearance and specific organ uptake of these complexes by injecting them into mice.

MATERIALS AND METHODS

Buffers. PBS: isotonic phosphate-buffered saline, pH 7·4; EDTA-GPB: PBS containing 0·1% gelatin and 10 mM EDTA; BSA-Dulbecco's PBS: Dulbecco's PBS (GIBCO, Grand Island, New York, USA) containing 0·5% BSA (Sigma), 0·1 mM $CaCl_2$, 0·5 mM $MgCl_2$, penicillin (100 u/ml) and streptomycin (100 μg/ml); HBSS: Hanks' balanced salt solution (GIBCO) containing penicillin and streptomycin; BSA-RPMI 1640: RPMI 1640 (GIBCO) containing 0·5% BSA, penicillin and streptomycin.

*Present address: Department of Bacteriology, Osaka University Medical School, Suita, Osaka 565, Japan.

Correspondence: Dr Teizo Fujita, Department of Immunology, Institute of Basic Medical Sciences, University of Tsukuba, Niihari-gun, Ibaraki-ken 305, Japan.

Antisera. Rabbit antiserum to ovalbumin (OV) was obtained by immunizing a New Zealand rabbit with OV (Grade V, Sigma). IgG antibody was isolated by affinity chromatography. Labelling of IgG with ^{125}I was performed using IODO-GEN, (Pierce). The specific activity was $1–6 \times 10^6$ ct/min/μg protein. Monoclonal antibody (MoAb) to C3bi receptor (anti-Mac-1) was purchased from Hybritec Incorporated, San Diego, California, USA.

Immune precipitates. Immune precipitates were prepared at equivalence with OV and radiolabelled IgG antibody to OV. The mixture was incubated at 37°C for 30 min, then at 4°C overnight. The resulting precipitates were washed three times and resuspended in PBS.

Solubilized complexes. Immune precipitates containing 1–10 μg of IgG were incubated with 1 ml of mouse serum at 37°C for 1 h. Twenty-five microlitres of the mixture were drawn, diluted with 2 ml of cold EDTA-GPB, and centrifuged at 5,000***g*** for 15 min. Both pellets and supernates were assayed for radioactivity, and a percent solubilization was calculated. The residual reaction mixture was centrifuged at 5,000***g*** for 15 min at 4°C. The supernate was used for the experiments.

Antigen excess immune complexes. OV and its IgG antibody were mixed at five-fold antigen excess, incubated at 37°C for 1 h and at 4°C overnight. The precipitates were removed by centrifugation at 5,000***g*** for 15 min, and the supernate was used.

Mouse peritoneal macrophages (MPM). C57Bl/6 male mice were injected i.p. with 2 ml of sterilized 5% Bactothioglycollate broth (DIFCO). Four to five days after injection, the mice were sacrificed and the peritoneal cells were harvested by injecting 5 ml of cold HBSS containing 1 unit of heparin/ml and by withdrawing the fluids. The cells were centrifuged, washed twice and suspended in BSA-RPMI 1640 at a concentration of $1{\cdot}5 \times 10^7$ cells/ml. Greater than 80% of the peritoneal cells were esterase positive with α-naphtyl butyrate substrate.

Aggregated IgG. Rabbit IgG was purified from 40% ammonium sulphate precipitate of normal rabbit serum by DEAE cellulose chromatography. The rabbit IgG was aggregated by heating for 20 min at 65°C.

Adherence of immune complexes to MPM. Radiolabelled immune complexes were added to MPM and incubated under 5% CO_2 and 95% air. After incubation, the reaction was stopped by diluting each tube with 1 ml of ice cold BSA-Dulbecco's PBS. The tubes were centrifuged at 1,500***g*** for 5 min and washed again. Trichloroacetic acid (TCA) was added to the collected supernatants at a final concentration of 10%, and separated into TCA precipitable and TCA non-precipitable fractions. Radioactivities of the cells and the fractions were measured, corrected for radioactivity of the control tubes which were composed of radiolabelled immune complexes and buffer, and expressed as a percentage of the original amount of radiolabelled complexes added to the cells.

In vivo *blood clearance of immune complexes.* Solubilized complexes, antigen excess immune complexes, or antibody alone each containing $1{\cdot}5$ μg (5×10^5 ct/min) of IgG antibody in a volume of 200 μl was injected into the tail vein of mice. Subsequently, 10 μl blood samples were taken from the tail artery after various intervals and expelled into 0·5 ml of EDTA-GPB. After addition of 1 ml of 15% TCA, samples were centrifuged and the radioactivities of the pellets were determined. The activity was expressed as a percentage of the initially injected radioactivity and plotted against time.

Specific organ uptake of solubilized complexes. Radiolabelled solubilized complexes or antibody alone was injected as described above. At selected time, the animal was sacrificed, and the amounts of the radioactivity within the liver, spleen, kidney, and lung were determined. The radioactivity of IgG alone within the organ was subtracted from the radioactivity of solubilized complexes in each organ to give a corrected value for the amounts of solubilized complexes bound within these organs. The amounts of ^{125}I within these organs were expressed as percentages of the total amount injected.

Radioautography. ^{125}I-labelled solubilized complexes containing 0·4 μg of antibody (2×10^5 ct/min) were i.v. injected into mice which were then sacrificed at 20 min. The liver was perfused with 1% glutaraldehyde in PBS, post-fixed in 1% osmium tetroxide in 0·1 M cacodylate-HCl buffer, pH 7·2, containing 7·5% sucrose, and embedded in Epon mixture. Two and half a micrometre sections were prepared for light and electron microscopic autoradiography, respectively. These sections were dipped into NR-M2 autoradiographic emulsion (Konishiroku Photo Ind. Co., Japan) and allowed to remain for 4 weeks at 4°C. The slides were then developed with D 19 (Kodak), fixed with Rapid fixer (Kodak), and stained lightly with 1% toluidine blue for light microscopy and with lead citrate and uranyl acetate for electron microscopy.

RESULTS

Phagocytosis and degradation of solubilized complexes and antigen excess immune complexes by MPM

When incubated with MPM at 37°C, solubilized complexes and antigen excess immune complexes bound to MPM in a similar fashion (Fig. 1). The degradation of both complexes proceeded linearly with time. In contrast to antigen excess immune complexes, however, solubilized complexes did not bind to MPM at 4°C. From these results, it is possible to conclude that the receptors on MPM for solubilized complexes are different from Fc receptors via which the antigen excess complexes bind, because the binding to Fc receptor is temperature-independent (Segal & Hurwitz, 1977). Further evidence for this conclusion is shown in Fig. 2. Even in the presence of a large excess of aggregated IgG, the percentage inhibition of the binding to MPM of solubilized complexes was around 20%, whereas that of antigen excess immune complexes was 70%. These results also indicate that the solubilization process inhibits the binding of immune complexes to Fc receptor. Since some complement components are assembled on immune complexes during solubilization (Takahashi *et al*, 1978; Fujita, Takata & Tamura, 1981) and the binding to complement receptor is temperature-dependent (Eden, Bianco & Nussenzweig, 1973), complement receptors on MPM could mediate the adherence of solubilized complexes.

Adherence of immune complexes to C3 receptors

We studied the adherence of immune complexes to C3b receptor (CR1) on human erythrocytes at various stages of solubilization (Fig. 3). The advantage of using human erythrocytes is that they only have CR1, whereas non-primate erythrocytes do not possess this receptor (Nelson, 1963).

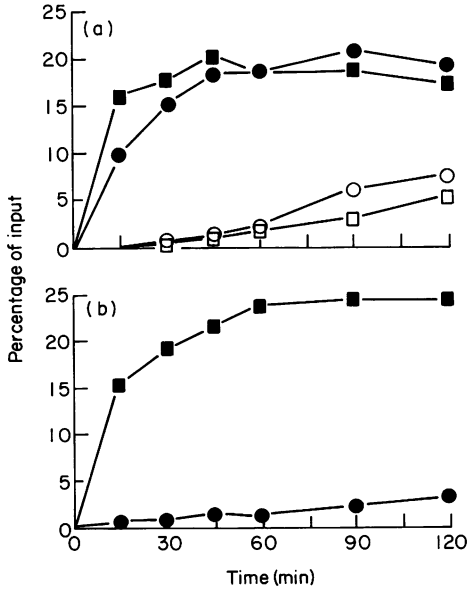

Fig. 1. Kinetics for phagocytosis and degradation of solubilized complexes and antigen excess immune complexes by MPM. Immune complexes (10 μl) containing 10 ng of radiolabelled antibody were added to 200 μl of cell suspension (3×10^6) and incubated at (a) 37°C or (b) 4°C for various times. The cells were washed and counted for the radioactivity (cell associated complexes). Catabolism of immune complexes was measured by counting the supernatant after acid precipitation (catabolized complexes). In control, ^{125}I-IgG alone did not bind to MPM (less than 3%). ●, cell associated solubilized complexes; ○, catabolized solubilized complexes; ■, cell associated antigen excess immune complexes; □, catabolized antigen excess immune complexes. Each determination represents the mean of triplicate samples.

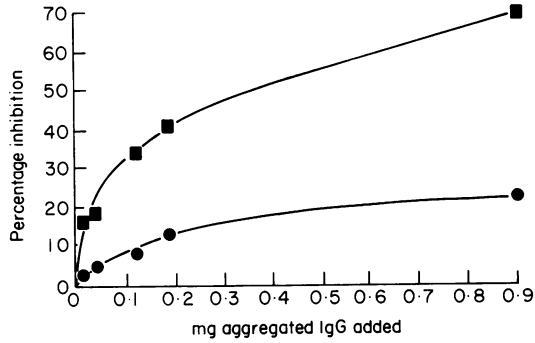

Fig. 2. Effect of aggregated IgG on binding of immune complexes to MPM. Increasing amounts of aggregated IgG were added to MPM (3×10^6), and incubated with immune complexes containing 10 ng of antibody for 1 h at 37°C in a volume of 210 μl. Cell associated immune complexes were assayed as described in the Materials and Methods. The amounts of immune complexes associated with MPM in control tubes without aggregated IgG were regarded as 100% adherence, and the percentage inhibition of adherence of complexes was calculated. ●, solubilized complexes; ■, antigen excess immune complexes. Each determination represents the means of triplicate samples.

Immune complexes at an early stage of solubilization acquired binding affinity for the Cr1, but this affinity was abolished when the solubilization process was completed. As reported elsewhere (Takata, 1984), the C3b bound to immune complexes is cleaved into C3bi by factors H and I during the solubilization process. Therefore, it seems possible that the binding of solubilized complexes to MPM is mediated by the C3bi receptor (CR3).

To examine this possibility, solubilized complexes were incubated with MPM in the presence of antibody to CR3 (anti-Mac-1). As shown in Fig. 4, anti-Mac-1 inhibited the binding of solubilized complexes to MPM by about 50%. Therefore, the binding of solubilized complexes to MPM seems to be mediated mainly by CR3. The inhibition was enhanced by addition of aggregated IgG, indicating that the Fc receptor on MPM was also responsible for the binding.

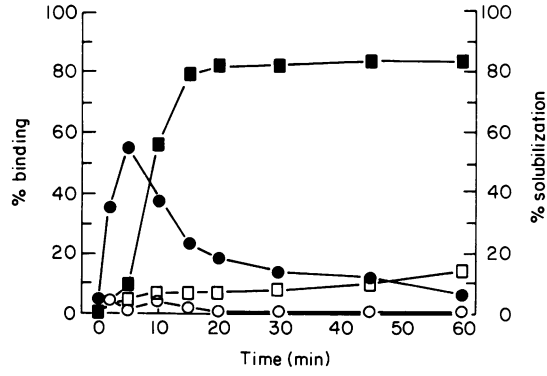

Fig. 3. Adherence of immune complexes to CR1 at various stages of solubilization process. Two aliquots of 15 μl of the immune precipitates containing 3 μg complexed antibody were incubated at 37°C with 3·0 ml of 1:3 dilution of fresh C57Bl/6 mouse serum (●, ■) or heated serum (56°C, 30 min) containing 10 mM EDTA (○, □). At various time intervals, 50 μl samples were drawn, diluted with 2 ml of cold EDTA-GPB and centrifuged to determine percentage solubilization (□, ■). At the same time, 100 μl samples were transferred to the second reaction mixture containing 300 μl suspension of human erythrocytes (8×10^7) in EDTA-GPB. The mixtures were incubated at 37°C for 45 min. Then, a 200 μl portion of the reaction mixture was diluted by 300 μl of EDTA-GPB, overlayed on 1·5 ml of Ficoll-Hypaque and centrifuged at 1,500 r/min for 5 min. Supernatants and pellets were assayed for radioactivity, and the percentage binding (○, ●) was determined. Each determination represents the mean of triplicate samples.

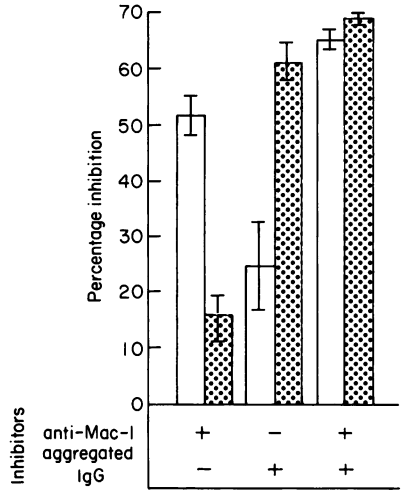

Fig. 4. Effect of antibody to CR3 (anti-Mac-1) on the binding of immune complexes. MPM (3×10^6 cells) were pre-incubated with either anti-Mac-1 (2 μg/ml) or aggregated IgG (2·5 mg/ml), or both for 15 min at 37°C in 5% CO_2. MPM were also preincubated with rabbit IgG (2 μg/ml) or buffer as controls. The MPM were then incubated for 20 min with 10 μl of ^{125}I-labelled solubilized complexes (□) or antigen excess soluble complexes (▨) containing 10 ng antibody. The cells were washed and cell associated radioactivity was assayed. The results were expressed as a percentage inhibition of cell associated immune complexes. Each value is the mean of three samples.

In vivo *clearance and tissue localization of solubilized complexes*

Next, we injected solubilized complexes into mice, and studied their clearance and organ sequestration (Fig. 5). The solubilized complexes were removed rapidly from the circulation, whereas antigen excess immune complexes disappeared more slowly. At 3 min, 30% of the solubilized complexes was already found in the liver. The radioactivity in the liver reached 50% at 20 min. Subsequently, the concentration of solubilized complexes in the liver decreased. In contrast, less than 3% of the injected radioactivity was found within the spleen, kidney and lung at any time. The localization of the solubilized complexes within the liver was determined by autoradiography. The solubilized complexes were located primarily within the Kupffer cells (Fig. 6 arrows).

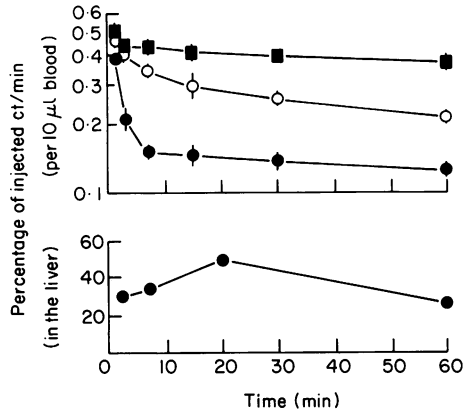

Fig. 5. Clearance of solubilized complexes and specific uptake by the liver. Upper panel shows blood clearance of solubilized complexes (●), antigen excess soluble complexes (○) and IgG antibody alone (■). Lower panel shows hepatic uptake of solubilized complexes. Data points represent the means of at least three mice.

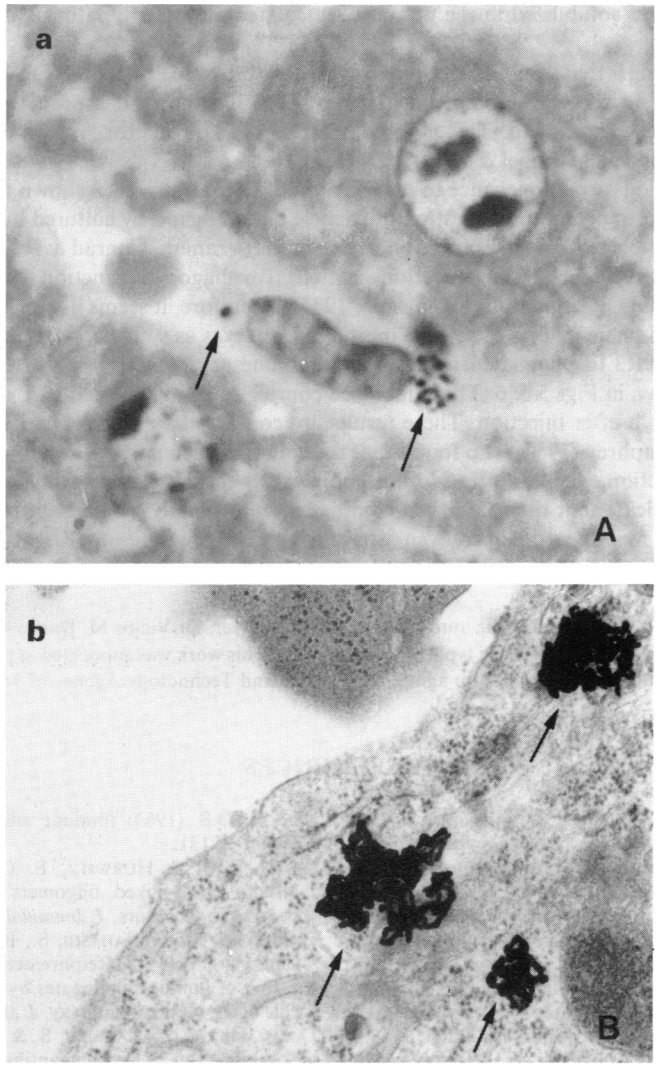

Fig. 6. Autoradiography of the liver 20 min after injection of solubilized complexes. (a) Light microscopy. Magnification × 2,150. (b) Electron microscopy. Magnification × 16,000. Arrows indicated the ingested solubilized complexes.

DISCUSSION

The main finding of the present paper is that the solubilized complexes are capable of binding to MPM mainly though CR3 in spite of the loss of binding to CR1 and that they were rapidly cleared from the circulation and captured by the Kupffer cells in the liver.

Previous reports have shown that the solubilization process inhibits the binding of immune complex to CR1 and Fc receptors of various blood cells (Eden *et al.*, 1973; Michl *et al.*, 1979; Takahashi *et al.*, 1980). We now show that solubilized complexes retain the binding affinity for MPM mainly through CR3, although solubilized complex was considered to be 'dead-end' complex incapable of binding to membrane receptors any longer (Takahashi *et al.*, 1980). At that time CR3 is not well characterized. This finding agrees with our recent report (Takata *et al.*, 1984) showing that

immune aggregates solubilized in the presence of isolated alternative pathway components bear C3bi.

Another finding is that solubilized complexes do not bind to MPM at 4°C (Fig. 1). As mentioned above, their binding to MPM is mainly mediated by CR3. Therefore, it appears that the binding to CR3 is temperature-dependent. This is in contrast to the Fc receptor, via which the binding is temperature-independent (Segel & Hurwitz, 1977).

Our observations are of relevance to the function of CR3. It has been shown that CR3 is more efficient than CR1 in promoting ingestion of particle bound ligand by cultured human monocytes (Wright & Silverstein, 1982) and rat peritoneal mast cells (Vranian, Conrad & Ruddy, 1981). Also, patients with severe life threatening infection and defective phagocytic function show defective CR3 activity but normal CR1 function (Dana *et al.*, 1984). Therefore, it seems likely that CR3 plays an important role in phagocytosis of soluble immune complexes as well as particle antigens.

The role of CR3 in phagocytosis of soluble immune complexes is supported by the *in vivo* experiments shown in Figs 5 & 6. The solubilized complexes were captured by the Kupffer cells in the liver within 1 h after injection. These results are consistent with the experiments of Malasit, Bartolotti & Hamphrey (1983) who found that the solubilized complexes accumulated in the liver shortly after injection, whereas after 24 h the complexes were taken up and retained in the spleen. These results indicate that the solubilized complexes are probably catabolized by the reticuloendothelial system, mainly in the liver, even after having lost the affinity for CR1 and Fc receptors. This process is most likely mediated by CR3.

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REFERENCES

DANA, N., TODD, R.F., PITT, J., SPRINGER, T.A. & ARNAOUT, M.A. (1984) Deficiency of a surface membrane glycoprotein (Mol) in man. *J. clin. Invest.* **73,** 153.

EDEN, A., BIANCO, C. & NUSSENZWEIG, V. (1973) Mechanism of binding of soluble immune complexes to lymphocytes. *Cell. Immunol.* **7,** 459.

FUJITA, T., TAKATA, Y. & TAMURA, N. (1981) Solubilization of immune precipitates by six isolated alternative pathway proteins. *J. exp. Med.* **154,** 1743.

MALASIT, P., BARTOLOTTI, S.R. & HUMPHREY, J.H. (1983) Molecular composition of complement-solubilized complexes and their fate *in vivo*. *Immunology*, **48,** 779.

MICHL, J., PIECZONKA, M.M., UNKELESS, J.C. & SILVERSTEIN, S.C. (1979) Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. exp. Med.* **150,** 607.

MILLER, G.W. & NUSSENZWEIG, V. (1975) A new complement function: solubilization of antigen–antibody aggregates. *Proc. Natl. Acad. Sci. USA.* **72,** 418.

MILLER, G.W., SALUK, P.H. & NUSSENZWEIG, V. (1973) Complement-dependent release of immune complexes from the lymphocyte membrane. *J. exp. Med.* **138,** 495.

NELSON, D.S. (1963) Immune adherence. *Adv. Immunol.* **3,** 131.

SEGAL, D.M. & HURWITZ, E. (1977) Binding of affinity cross-linked oligomers of IgG to cells bearing in receptors. *J. Immunol.* **118,** 1338.

TAKAHASHI, M., TAKAHASHI, S., BRADE, V. & NUSSENZWEIG, V. (1978) Requirements for the solubilization of immune aggregates by complement. The role of the classical pathway. *J. clin. Invest.* **62,** 349.

TAKAHASHI, M., TAKAHASHI, S. & HIROSE, S. (1980) Solubilization of antigen–antibody complexes. A new function of complement as a regulator of immune reactions. *Prog. Allergy*, **27,** 134.

TAKATA, Y., TAMURA, N. & FUJITA, T. (1984) Interaction of C3 with antigen–antibody complexes in the process of solubilization of immune precipitates. *J. Immunol.* **132,** 2531.

VRANIAN, G., CONRAD, D.H. & RUDDY, S. (1981) Specificity of C3 receptors that mediate phagocytosis by rat peritoneal mast cells. *J. Immunol.* **126,** 2302.

WRIGHT, S.D. & SILVERSTEIN, S.C. (1982) Tumor-promoting phorbol esters stimulate C3b and C3b′ receptor-mediated phagocytosis in cultured human monocytes. *J. exp. Med* **156,** 1149.