Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 3816–3820, June 1983
Medical Sciences

Cytolysis of nucleated cells by complement: Cell death displays multi-hit characteristics

(membrane attack complex/cytotoxicity/rubidium release)

CAROL L. KOSKI*, LOUISE E. RAMM†, CARL H. HAMMER‡, MANFRED M. MAYER†, AND MOON L. SHIN§¶

Departments of §Pathology and *Neurology, University of Maryland School of Medicine, Baltimore, Maryland 21201; †Department of Molecular Biology and Genetics, Subdepartment of Immunology, The Johns Hopkins Medical School, Baltimore, Maryland 21205; and ‡Laboratory of Clinical Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

Contributed by Manfred M. Mayer, March 22, 1983

ABSTRACT **Lysis of nucleated cells by complement was studied to determine whether the lytic process by C5b-9 conforms to a one-hit mechanism as in the case of erythrocytes. Two nucleated cell lines, Molt 4 and U937, derived from human T lymphocytes and histiocytes, respectively, were employed as targets. The antibody-sensitized cells were used to develop the titration curves, measuring cell death as a function of limiting quantities of human C6 or C5,6 complex in the presence of an excess of other complement components. The cytolysis curves generated in both experiments were sigmoidal, in sharp contrast to the monotonic curves observed in lysis of erythrocytes treated similarly. The sigmoidal curves of cytolysis indicate a cooperative action of several molecules of C6 or acid-activated C5,6 complex, C(56)^a. In contrast to the multi-hit characteristics of cytolysis, dose–response measurements of the release of ^{86}Rb indicated that only one effective molecule of C6 per cell is required for assembly of a ^{86}Rb-releasing channel. This divergence indicates that lysis requires formation of several channels or, alternatively, assembly of large channels that are formed by several molecules of C6. Because prior studies with erythrocyte ghosts have shown that only a single effective molecule of C6 is required for assembly of a transmembrane channel, regardless of size, we prefer to interpret the multi-hit characteristics of nucleated cell lysis as an indication of a multi-channel requirement, rather than channel enlargement.**

During the past decade, the mechanism of membrane attack by complement has been studied extensively with erythrocytes and artificial lipid bilayers as targets. The results of these investigations indicate that: (*i*) hydrophobic domains of complement proteins are exposed during sequential interaction of the terminal complement proteins, C5–C9 (1–5); (*ii*) these hydrophobic peptides become inserted in the lipid bilayer of membranes (6–10), a process that is followed by protein–lipid or protein–protein rearrangement or both (9–10); and, finally (*iii*), functional transmembrane channels are formed, resulting in colloid osmotic lysis of cells (11–15).

Since 1953 several lines of experimental information have been developed which demonstrate that the lysis of erythrocytes by complement is a one-hit process—i.e., that one channel suffices for lysis of an erythrocyte (16–20). The most convincing evidence came from dose–response curves for C5b6, C7, C8, and C9 (21–23). As a consequence of the development of the one-hit concept, the quantitative relationship between the formation of complement lesions and cell lysis has been clarified and formulated in mathematical terms (18). Also, the one-hit theory played a leading part in the formulation of the doughnut hypothesis of membrane attack by complement (24).

There are several observations in prior publications that can be construed as possible indications that the cytocidal action of complement against nucleated cells may not be a one-hit process (25–27). This suggestion may also explain the well-known fact that nucleated cells are known to be far more resistant to complement-mediated killing than are erythrocytes. In an effort to elucidate this issue, Ohanian, Schlager, and Borsos have made extensive biochemical studies of complement attack on nucleated cells, and they have presented the concept that these cells are relatively resistant because they possess mechanisms of defense against complement attack (28–30). Specifically, they reported a rise in lipid synthesis that occurs within minutes of attack by complement and they presented experiments on certain drugs and hormones that affect lipid metabolism as well as susceptibility to complement. It is evident from these studies that a linkage exists between cell metabolism, notably membrane lipid synthesis, and resistance to complement, but the nature of this relationship is unclear. As an initial step towards the development of mechanistic information on the action of complement against nucleated cells, we have performed experiments designed to determine whether the lytic process conforms to a one-hit mechanism. As shown in the present paper, we found that the lysis of nucleated cells is a multi-hit process, at least in the case of two cell types of human origin: Molt 4, a thymus-derived cell, and U937, a macrophage-like cell line.

MATERIALS AND METHODS

Buffers. VBS: Veronal (barbital)-buffered saline, pH 7.4 (145 mM NaCl/5 mM sodium Veronal), was prepared by diluting a stock solution 5-fold with water (31). GVB^{++}: VBS containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$. $DGVB^{++}$: 75 mM NaCl VBS, pH 7.4, containing 25% dextrose, 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$. RPMI buffer: RPMI 1640 medium containing 25 mM Hepes (Sigma) and 2% bovine serum albumin, pH 7.4. EDTA buffer: Hanks' balanced salt solution (GIBCO) containing 20 mM EDTA.

Radioisotopes. ^{86}RbCl (1.31 mCi/mg of rubidium; 1 Ci = 3.7×10^{10} Bq) was purchased from New England Nuclear. Prior to use, the required quantity of radioactive RbCl solution was diluted in phosphate-buffered saline, pH 7.4, and its activity

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: C, complement (complement components are designated by number—i.e., C1, C2, . . .—and the fragments of the component are designated by small letter—e.g., C3a or C3b); C(56)^a, a C5,6 complex activated by acidification of C5 and C6; C6D-RS, rabbit serum from animals congenitally deficient in C6; VBS, Veronal-buffered saline, pH 7.4; GVB^{++}, VBS containing gelatin, $CaCl_2$, and $MgCl_2$; $DGVB^{++}$, 75 mM NaCl VBS containing dextrose, gelatin, $CaCl_2$, and $MgCl_2$; NA, nucleated mammalian cells sensitized with specific antibody; EA, mammalian erythrocytes sensitized with specific antibody; LDH, lactate dehydrogenase.

¶To whom reprint requests should be addressed.

was measured in a Packard Autogamma Scintillation Spectrometer (the final solution contained 1.75×10^6 cpm/ml). In the experiments proper, the radioactivity of ^{86}Rb was measured as β emission in a Beckman LS-233 liquid scintillation counter.

Complement and Complement Components. Pooled neonatal rabbit serum was purchased from Pel-Freez Biologicals. Rabbit serum from animals congenitally deficient in C6 (C6D-RS) was the gift of C. Rother (University of Heidelberg). Human C5 and C6 were purified according to the method described in ref. 32. The hemolytic titers of C5 and C6 were 7.4×10^5 and 9×10^4 units/ml, respectively. The acid-activated C5,6 complex C(56)^a was prepared with purified human C5 and C6 as described in ref. 33. The two components were brought to pH 6.4 at 0°C with 0.15 M HCl, then the pH was brought to 7.4 with 0.2 M NaOH.

Target Cells. Molt 4 cells, a human T cell lymphoma line, and U937 cells derived from a human histiocytic leukemia were maintained in suspension culture in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories) and 2 mM glutamine (MA Bioproducts, Walkersville, MD). Gentamycin sulfate (Schering) and amphotericin B (GIBCO), 50 and 0.25 μg/ml, respectively, were used in Molt 4 cell cultures; 100 units of penicillin per ml and 100 μg of streptomycin per ml (GIBCO) were used in cultures of U937 cells. Sheep erythrocytes were also used as a target cell, as described in ref. 32.

Antibodies. A hybridoma-derived mouse monoclonal IgG antibody against Molt 4 cells and a monoclonal IgM antibody against U937 cells were used to sensitize Molt 4 and U937 cells, respectively. These antibodies are generous gifts from Robert Johnson (The Johns Hopkins Medical School).

Complement-Mediated Nucleated Cell Death. Molt 4 cells were centrifuged at $200 \times g$ for 5 min at 4°C in an IEC International centrifuge. Cells were washed twice in VBS and suspended to 4×10^6 cells per ml in GVB^{++}. Cells were incubated with an equal volume of an optimal antibody dilution (see Table 1) for 30 min at 37°C in a shaking water bath. The antibody-coated nucleated cells (NA) were washed twice in GVB^{++} and suspended to 2×10^6 per ml in GVB^{++}. One hundred microliters of NA was incubated with 100 μl of rabbit serum of various dilutions at 37°C for 90 min. The cell viability was determined.

Assays for Cell Viability. *Vital dye exclusion method.* One hundred microliters of each of the final reaction mixtures were mixed with 100 μl of 0.4% Sudan black or 0.4% trypan blue. The positively staining cells were expressed as percent of the total number of cells counted in a hemocytometer as described (34). All determinations were done in duplicate or triplicate. Parallel determinations of cell death were made by vital dye exclusion and lactate dehydrogenase (LDH) release. The positive correlation between the two methods would be important because it is not known whether large complement channels could allow vital dye to enter the cells in the absence of cell lysis. The release of LDH (molecular weight 135,000) is a good indication of cell lysis because the enzyme is too large to diffuse out via the largest complement channels (14). *LDH method.* In this assay, the optimal concentration of NA used was 2×10^7 cells per ml. Cells were centrifuged in a Beckman Microfuge at 4°C and the supernates were assayed for LDH according to the protocol supplied with the Sigma diagnostic kit for LDH with minor modification. Four hundred microliters of NADH (6 mM NADH in 500 mM sodium phosphate buffer, pH 7.4) was mixed with 400 μl of a 5-fold or 10-fold dilution of supernate in a 1-ml cuvette. To this, 200 μl of 6 mM sodium pyruvate was added with immediate mixing. The absorbance was measured at 340 nm in a Gilson UV spectrophotometer. The 100% LDH activity in the cell was obtained by adding 2 μl of melittin (5 mg/ml) to solubilize 2×10^6 cells. Solubilization of cells with sodium dodecyl sulfate or deoxycholate was unsatisfactory due to their interference with the LDH assay. Repeated freezing and thawing did not fully release intracellular LDH into the medium.

Preparation of Cells Carrying C3b. Antibody-sensitized Molt 4 cells (4×10^6 per ml) in DGVB^{++} were incubated with an equal volume of C6D-RS (1:2 final dilution) for 30 min at 37°C. Cells were washed twice with GVB (VBS containing 0.1% gelatin) and further incubated at 37°C for 60 min in GVB containing 10 mM EDTA to dissociate C1-rs, C2a, and C5b (35). The presence of C3b was checked by rosette formation with human erythrocytes according to ref. 36. More than 80% of the Molt 4 cells were adherent to human erythrocytes. Sheep erythrocytes carrying C3b (EAC3b) were made according to ref. 35.

Lysis of NAC3b or EAC3b by C(56)^a and C7–C9. For lysis of NAC4b3b cells, the mixture of 5×10^4 units each of C5 and C6 was acidified and then neutralized to pH 7.4 according to ref. 33. The total volume was adjusted to 1.0 ml with DGVB^{++}. Fifty microliters of NAC4b3b (8×10^6 cells per ml) was incubated with 150 μl of C(56)^a of various concentrations for 10 min at 27°C, followed by reaction with 50 μl of C6D-RS (1:32 final dilution) for an additional 90 min at 37°C. The cell viability was examined by the vital dye exclusion method. In parallel experiments with erythrocyte targets, the lysis of EAC4b,3b cells with C(56)^a was carried out as described in ref. 33 except that a 1:27 dilution of C6D-RS was used instead of guinea pig serum with 10 mM EDTA as a converting reagent for C(56)^a.

Incorporation of ^{86}Rb into Cells. U937 cells were washed twice and resuspended in a total volume of 1.0 ml in RPMI buffer to a concentration of 1.0×10^7 cells per ml. Small quantities (50–60 μl) of ^{86}Rb were added and the cells were incubated for 1 hr at 37°C with shaking. At the end of this period, extracellular ^{86}Rb was removed by layering the cells onto 2.0 ml of heat-inactivated newborn calf serum (MA Bioproducts) and centrifuging for 1 min at maximal speed in a table-top centrifuge (International clinical centrifuge). The cell pellets were resuspended in 1.0 ml of RPMI buffer and the washing procedure was repeated twice. The final cell pellet was resuspended in RPMI buffer to a concentration of 3.0×10^6 cells per ml (the cell suspension contained approximately 2×10^5 cpm/ml as β emission).

Endpoint Experiments on ^{86}Rb-Labeled Cells Treated with Antibody and Complement. One volume of ^{86}Rb-containing U937 cells (3×10^6 cells per ml) was incubated for 30 min at 27°C with an equal volume of 1:200 dilution of antibody (this dilution was chosen for maximal release, based on preliminary tests). The cells were centrifuged once ($200 \times g$) for 3 min at 4°C in an IEC International centrifuge and resuspended to 2×10^6 cells per ml in RPMI buffer. Aliquots (100 μl) of the antibody-treated cells were mixed with 50 μl of C6D-RS (undiluted) and 50 μl of various dilutions of C6.

One set of samples was incubated for 25 min at 37°C. This time was sufficient to reach the endpoint of ^{86}Rb release (data not shown). At the end of the incubation, 200 μl of ice-cold EDTA buffer was added to each sample and the cells were centrifuged at $200 \times g$ for 3 min at 4°C. Release of ^{86}Rb was determined by measuring radioactivities of aliquots of the supernatant fluid, as well as the residual pellet. The total amount of ^{86}Rb in untreated cells was determined by lysing the cells with 2 μl of melittin at 5 mg/ml, centrifuging, and measuring the released ^{86}Rb. All of the intracellular ^{86}Rb was released by this method. A second set of samples (cells treated with antibody, C6D-RS, and C6, as indicated above) was incubated for

Table 1. Effect of IgG antibody concentration on lysis of Molt 4 cells with complement*

	Antibody concentration × 10^6							
	0	0.125	0.25	0.5	1.0	2.0	4.0	20.0
% cells stained by vital dye	3	4.5	8	23	60	82	97	100

*Fresh neonatal rabbit serum was used as a source of whole complement in a final dilution of 1:4.

2 hr at 37°C. At the end of this incubation, cell viability was assessed. One hundred microliters of the cells was mixed with 100 μl of 0.4% trypan blue and the number of cells stained by vital dye was determined.

RESULTS

Determination of Antibody Concentration in the Lysis of Nucleated Cells by Complement. Appropriate antibody concentrations were determined with a constant dose of rabbit complement (25% final concentration) and a fixed number of Molt 4 cells (2×10^5). As shown in Table 1, 100% cell death estimated by staining with vital dye was obtained with a final antibody concentration of 2×10^{-5}. All subsequent assays were performed with a constant dose of antibody equal to twice that concentration. No significant agglutination was observed.

Kinetics of Cytolysis Assayed by Trypan Blue Uptake and LDH Release. Molt 4 cells sensitized with antibody were incubated at 37°C with various dilutions of rabbit serum complement or with 25% C6D-RS plus various quantities of purified C6. Samples were taken at different time points, and the degree of lysis was analyzed by vital dye uptake (Fig. 1 *A* and *B*) and by measurement of LDH released into the supernates (Fig. 1*A*). Fig. 1 *A* and *B* shows the kinetics of cell death in relation to the dose of whole serum complement or C6, respectively.

The results of these kinetic experiments indicate that the endpoint plateau was reached at about 60 min regardless of complement dose in the experiments with rabbit serum complement (Fig. 1*A*), whereas approximately 90 min was required to reach the endpoint in the experiments with C6D-RS and purified C6 (Fig. 1*B*). This difference is due to the 10-fold higher cell concentration that was used in Fig. 1*A* to satisfy the requirements of the LDH assays. In assessing cell viability, the results obtained with the vital dye exclusion method were identical with those obtained by measuring released LDH, a soluble protein marker in the cytoplasm. The extent of LDH release into the medium was the same whether the supernates were obtained by centrifuging cells at 500 × *g* or 25,000 × *g* at 4°C.

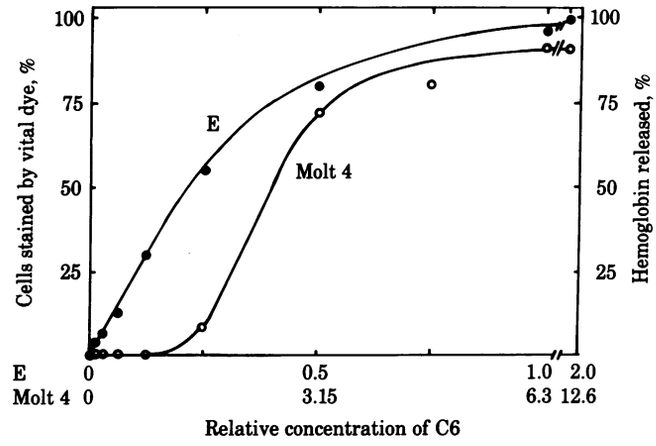

FIG. 2. C6 dose response of nucleated cell or erythrocyte lysis. Molt 4 cells coated with IgG antibody were incubated for 90 min at 37°C with excess C6D rabbit serum (1:4 final dilution) and limiting concentrations of human C6. Cell death was measured by vital dye uptake. A sigmoidal dose–response curve was obtained with nucleated cells (○) in contrast to the linear dose response characteristics of a parallel experiment with antibody-sensitized erythrocytes (●). The relative C6 concentration of 1.0 = 63 units of hemolytic activity and 323 ng of protein.

Cytolytic Dose–Response Curves with Various Concentrations of C6. Molt 4 cells or sheep erythrocytes can be lysed by C6D-RS plus purified human C6 to the same extent as by a comparable concentration of normal rabbit serum. C6 dose–response curves for Molt 4 cells and sheep erythrocytes in the presence of excess C6D-RS are shown in Fig. 2. The dose–response curve of Molt 4 cells was sigmoidal, whereas the lysis of IgM antibody-coated erythrocytes displayed a monotonic relationship to the C6 concentration, with the line rising straight from the origin. Thus, the dose–response curve in Molt 4 cell killing indicates cooperative behavior of C6 molecules in the lytic process, whereas the erythrocyte dose–response curve displays one-hit characteristics, as expected. Furthermore, much more C6 was required for lysis of Molt 4 cells than for lysis of erythrocytes.

Cytolytic Dose–Response Curves of Molt 4 Cells and Erythrocytes with Various Doses of C(56)[a]. This experiment is essentially comparable to the previous one, except that preformed C(56)[a] was used for developing the dose–response relationship instead of purified C6. Cells carrying C3b were used

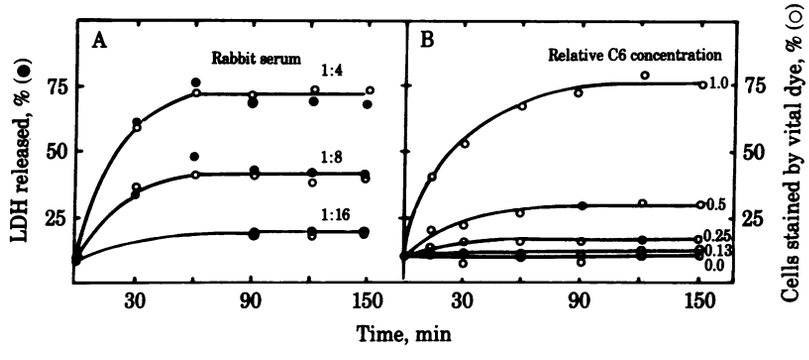

FIG. 1. Kinetics of nucleated cell lysis with limiting doses of serum complement or C6. (*A*) Molt 4 cells, sensitized with IgG monoclonal antibody, were incubated with limiting concentrations of rabbit serum at 37°C. Aliquots of cells (2×10^6) were taken at different time intervals. LDH release was measured in the supernates (●), and the cells were stained with vital dye (○). (*B*) Molt 4 cells coated with IgG antibody were treated with C6D-RS (1:4 final dilution) reconstituted with limiting doses of human C6. Aliquots of cells (2×10^5) were taken at various time intervals. Cells were stained with vital dye (○). The relative C6 concentration of 1.0 = 150 units of hemolytic activity and 807 ng of protein.

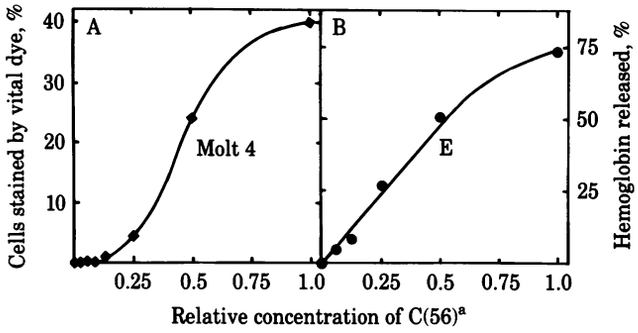

FIG. 3. C(56)ª dose–response curve for cytolysis of nucleated cells or erythrocytes carrying C3b. Molt 4 cells carrying C3b were made with C6D-RS (1:2 final dilution) (see text). The cells were then incubated with various concentrations of C(56)ª for 10 min at 27°C, followed by further incubation with C6D-RS (1:32 dilution) for 90 min at 37°C. Cells were stained with vital dye (*A*). Erythrocytes carrying IgM antibody and C3b were similarly treated (*B*). The relative C(56)ª concentration of 1.0 = 4,200 units each of C5 and C6 or 6.8 and 15 μg of protein, respectively, in *A* and 200 units each of C5 and C6 in *B*. It is evident that a sigmoidal dose–response curve was observed with nucleated cells (*A*), in contrast to the monotonic nature of the erythrocyte lysis (*B*) in relation to C(56)ª concentration.

because the reaction of the membranes with C(56)ª is extremely inefficient in its absence. As shown in Fig. 3*A*, the dose–response curve of C(56)ª in nucleated cell lysis is also sigmoidal. In contrast, the lysis of sheep erythrocytes carrying C3b displayed one-hit characteristics, as expected (Fig. 3*B*).

Dose–Response Curves for ^{86}Rb Release and Cytolysis with Nucleated Cells. As shown in Fig. 4, formation of a ^{86}Rb-releasing channel in the membranes of U937 cells requires only one effective molecule of C6, even though lysis of these cells is a multi-hit process. It follows that lysis of U937, unlike that of erythrocytes (37), requires more ion flux than that produced by a single ^{86}Rb-releasing channel.

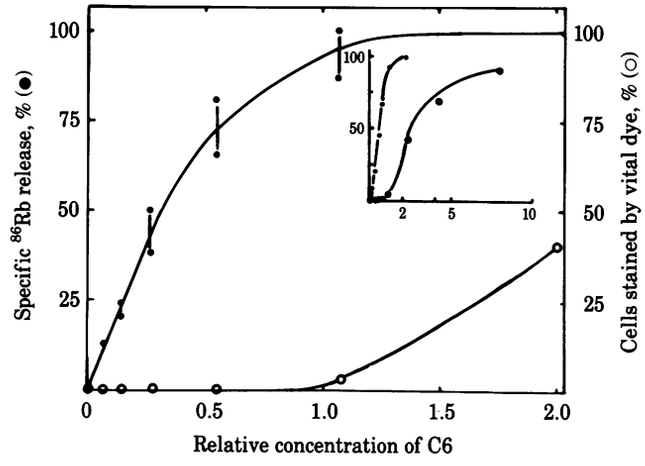

FIG. 4. C6 dose–response curve of ^{86}Rb release and lysis of nucleated cells. U937 cells loaded with ^{86}Rb were sensitized with IgM monoclonal antibody and incubated with excess C6D-RS (1:4 final dilution) and limiting concentrations of human C6. A set of cells was incubated for 25 min at 37°C and ^{86}Rb release was determined (●). The incubation of a second set of cells was continued for 2 hr, and the cells were stained with vital dye (○). The ^{86}Rb release from U937 cells showed a monotonic curve starting from the origin, as a function of C6, indicating channel formation with single-hit characteristics. Cell death, determined by vital dye staining, showed a sigmoidal curve, as shown in Figs. 2 and 3. (*Inset*) Entire dose–response curves. The relative C6 concentration of 1.0 = 32 units of hemolytic activity and 163 ng of protein.

DISCUSSION

In the present experiments we have used two approaches to determine whether the process of killing nucleated cells by complement is a one-hit or a multi-hit process. One of these involves the development of a dose–response curve with respect to C6, with excess of the other complement proteins supplied by C6-deficient serum. These experiments were performed at the endpoint of cell death with respect to time, as determined in prior kinetic experiments (Fig. 1). As shown in Fig. 2, the dose–response of sheep erythrocytes with respect to C6 produced a monotonic curve passing through the origin. By contrast, with Molt 4 cells as targets, the dose–response curve was sigmoidal, indicating multi-hit behavior. In the second experimental approach, dose–response curves were developed with respect to C(56)ª, which behaves in a manner closely similar to C5b6. In these tests, C6D-RS was used as a source of C7, C8, and C9. As shown in Fig. 3, the dose–response curve with erythrocyte targets indicated one-hit behavior, whereas the lysis of Molt 4 cells displayed multi-hit characteristics.

The sigmoidal shape of the lytic dose–response curves for nucleated cells (Figs. 2–4) means that several effective molecules of C6 or C(56)ª are needed for lysis of a single cell. By contrast, as shown in Fig. 4, only one effective molecule of C6 per cell is required for assembly of a ^{86}Rb-releasing channel. This divergence indicates, in agreement with ref. 25, that lysis of nucleated cells requires more ion flux than that produced by a single ^{86}Rb-releasing channel. This need could be met by formation of several channels; alternatively, the increase of ion flux could be produced by channel enlargement. Either of these options would be compatible with the data in Figs. 2–4.

Because information on the size of the complement channels in the membranes of nucleated cells is not yet available, the choice between these alternative interpretations must be based, for the present, on studies with erythrocyte ghosts. Specifically, it has been shown that complement channels in erythrocyte ghost membranes vary widely in size, ranging between 0.7 nm, or less, and about 7 nm in diameter (15), and that only a single molecule each of C5 and C6 is required for assembly of a transmembrane channel, regardless of size (23). Therefore, we prefer to interpret the multi-hit characteristics of nucleated cell lysis as a requirement for multiple channels, rather than channel enlargement.

Complement channels, when formed on erythrocyte membranes, are stable and allow ions to flow freely through the channel for extended periods of time (15). If the rate of flow through a single channel exceeds the rate of cation flux mediated by the membrane pumps, water will flow unchecked into the cell, causing it to swell and eventually to burst. The multi-hit characteristics of complement-mediated lysis in metabolically active nucleated cells might be due to the capacity of their membrane pumps to overcome the passive flux through a few complement channels. Alternatively, multiple complement channels may be required because channels in nucleated cells are unstable, as previously suggested (27). Because the metabolic activity of Molt 4 cells or U937 cells is much greater than that of erythrocytes, it is quite possible that either or both of these explanations may be applicable.

We thank Dr. Robert Johnson for his generous gifts of monoclonal antibodies and Dr. C. Rother for the C6-deficient rabbit serum. We appreciate Ms. E. Tinnell's help in preparing this manuscript. This work was supported by Multiple Sclerosis Society Grant RG 1374-A to M.L.S., U.S. Public Health Service Grant R03 DE06260 to C.L.K., and U.S. Public Health Service Grant R01 AI02566, Office of Naval Research Contract N00014-75-C-0142, and National Science Foundation Grant PCM 8012315 to M.M.M.

1. Inoue, K., Kinoshita, T., Okada, M. & Akiyama, Y. (1977) *J. Immunol.* **119,** 65–72.
2. Shin, M. L., Paznekas, W. A., Abramovitz, A. S. & Mayer, M. M. (1977) *J. Immunol.* **119,** 1358–1364.
3. Bhakdi, S., Bjerrum, O. J., Bhakdi-Lehnen, B. & Tranum-Jensen, J. (1978) *J. Immunol.* **121,** 2526–2532.
4. Podack, E. R. & Müller-Eberhard, H. J. (1978) *J. Immunol.* **121,** 1025–1030.
5. Podack, E. R., Biesecker, G. & Müller-Eberhard, H. J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 897–901.
6. Hammer, C. H., Nicholson, A. & Mayer, M. M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 5076–5080.
7. Bhakdi, S., Bjerrum, O. J., Rother, U., Knufermann, H. & Wallach, D. F. H. (1975) *Biochim. Biophys. Acta* **406,** 21–35.
8. Hammer, C. H., Shin, M. L., Abramovitz, A. S. & Mayer, M. M. (1977) *J. Immunol.* **119,** 1–8.
9. Hu, V. W., Esser, A. F., Podack, E. R. & Wisnieski, B. J. (1981) *J. Immunol.* **127,** 380–386.
10. Podack, E. R., Stoffel, W., Esser, S. F. & Müller-Eberhard, H. J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4544–4548.
11. Michaels, D. W., Abramovitz, A. S., Hammer, C. H. & Mayer, M. M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2852–2856.
12. Giavedoni, E. B., Chow, Y. M. & Dalmasso, A. P. (1979) *J. Immunol.* **122,** 240–245.
13. Boyle, M. D. P., Gee, A. P. & Borsos, T. (1979) *J. Immunol.* **123,** 77–82.
14. Ramm, L. E. & Mayer, M. M. (1980) *J. Immunol.* **124,** 2281–2287.
15. Ramm, L. E., Whitlow, M. B. & Mayer, M. M. (1983) *Mol. Immunol.* **20,** 155–160.
16. Mayer, M. M. (1953) *Atti VI Congr. Int. Microbiol.* **2,** 151–157.
17. Mayer, M. M. (1961) in *Immunochemical Approaches to Problems in Microbiology,* eds. Heidelberger, M. & Plescia, O. J. (Rutgers Univ. Press, New Brunswick, NJ), pp. 268–279.
18. Borsos, T., Rapp, H. J. & Mayer, M. M. (1961) *J. Immunol.* **87,** 310–325.
19. Hingson, D. J., Massengill, R. K. & Mayer, M. M. (1969) *Immunochemistry* **6,** 295–307.
20. Hoffman, L. G. (1969) *Immunochemistry* **6,** 309–325.
21. Yamamoto, K. & Gewurz, H. (1978) *J. Immunol.* **120,** 2008–2015.
22. Podack, E. R., Kolb, W. P. & Müller-Eberhard, H. J. (1978) *J. Immunol.* **120,** 1841–1848.
23. Ramm, L. E., Whitlow, M. B. & Mayer, M. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4751–4755.
24. Mayer, M. M. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2954–2958.
25. Boyle, M. D. P., Ohanian, S. H. & Borsos, T. (1976) *J. Immunol.* **117,** 1346–1350.
26. Reske-Kunz, A. B., Scheid, M. P., Abbott, J., Metakis, L. J., Polley, M. J. & Boyse, E. A. (1979) *Transplantation* **28,** 149–153.
27. Stephens, C. L. & Henkart, P. A. (1979) *J. Immunol.* **122,** 455–458.
28. Schlager, S. I., Ohanian, S. H. & Borsos, T. (1978) *J. Immunol.* **120,** 463–471.
29. Schlager, S. I., Ohanian, S. H. & Borsos, T. (1978) *J. Immunol.* **120,** 895–901.
30. Ohanian, S. H. & Schlager, S. I. (1981) *CRC Crit. Rev. Immunol.* **1,** 165–209.
31. Mayer, M. M. (1961) in *Experimental Immuno-Chemistry,* ed. Kabat, E. A. (Thomas, Springfield, IL), pp. 151–209.
32. Hammer, C. H., Wirtz, G. H., Reufer, L., Gresham, H. D. & Tack, B. F. (1981) *J. Biol. Chem.* **256,** 3995–4006.
33. Hänsch, G. M., Hammer, C. H., Mayer, M. M. & Shin, M. L. (1981) *J. Immunol.* **127,** 999–1002.
34. Absher, M. (1973) in *Tissue Culture Methods and Applications,* eds. Krase, P. F. & Patterson, M. K., Jr. (Academic, New York), p. 395.
35. Shin, H. S., Pickering, R. J. & Mayer, M. M. (1971) *J. Immunol.* **106,** 473–479.
36. Gaither, T. A., Hammer, C. H. & Frank, M. M. (1979) *J. Immunol.* **123,** 1195–1204.
37. Giavedoni, E. B., Chow, Y. M. & Dalmasso, A. P. (1979) *J. Immunol.* **122,** 240–245.