Killing of Human Melanoma Cells by the Membrane Attack Complex of Human Complement as a Function of Its Molecular Composition

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Abstract

The efficiency of the membrane attack complex (MAC) in killing M21 melanoma cells was determined varying the molar ratio of cell-bound C9:C8. It was found that (*a*) C5b-8 produced functional channels as evidenced by ^{86}Rb release and propidium iodide uptake; (*b*) cell killing occurred in the absence of C9 with $> 5 \times 10^5$ C5b-8/cell; (*c*) the maximal molar ratio of C9:C8 was 6.6:1; (*d*) using nonlytic numbers of C5b-8 (4.7×10^5/cell), > 90% killing ensued at a C9:C8 molar ratio of 2.8:1 at which ~ 9,000 poly C9/cell were formed, and 50% killing at a ratio of 1:1; (*e*) when the MAC was assembled on cells at 0°C, consisting of $C5b\text{-}8_19_1$, and unbound C9 was removed before incubation at 37°C, killing was similar to that observed when poly C9 formation was allowed to occur. Thus, MAC lytic efficiency toward M21 cells may be enhanced by but does not depend on poly C9 formation.

Introduction

Nucleated cells are capable of defense against attack by complement (1, 2). Prelytic (3, 4) and sublytic (5) doses of complement form functional channels on the plasma membrane of nucleated cells as measured by increased efflux of intracellular ^{86}Rb. Cytolytic inefficiency of these channels has been attributed to enhanced cellular metabolic activity (6–8) resulting in inactivation of complement channels by shedding (9) and internalization (10).

The rate of complement channel inactivation by nucleated cells has been attributed to qualitative differences in the membrane attack complex (MAC),[1] with progressive assembly of the MAC resulting in more rapid channel elimination (10). However, in these experiments, the molecular composition of the MAC and its correlation with lytic efficiency was not determined.

Although the relationship between MAC structure and function has been extensively studied with erythrocytes, no similar experiments have been performed with nucleated cells. Furthermore, it is unclear whether C5b-9 produces membrane damage through a detergentlike action, as set forth in the "leaky patch" theory (11–13), or through discrete hydrophilic transmembrane protein channels as proposed by the "doughnut" theory (14). Specifically, the role of poly C9, a circular structure containing 12–18 C9 molecules (15–17), in MAC lytic function is not resolved. Direct binding experiments using erythrocytes found C9:C8 molar ratios of 3–4:1 (18, 19), 6:1 (20), and 15:1 (17). Marked heterogeneity of MAC size and composition has been reported and related directly to the number of C9 molecules in the complex (21, 22). It was recently demonstrated that thrombin-cleaved C9, which is unable to form tubular poly C9, retained full hemolytic activity (23). Also, studies have shown varying degrees of lytic activity of complement in the absence of C9 in erythrocytes (24, 25), the nucleated cell line U937 (26), and in *Giardia lamblia* (27), indicating significant membrane labilization by C5b-8 complexes. In contrast, several nucleated cell lines appeared to be resistant to lysis by C5b-8 (4, 10).

The purpose of this study was to investigate the relationship between binding of the MAC proteins to the target membrane, causation of membrane damage, and cytolysis of the human M21 melanoma cell line. Evidence is presented showing that C5b-8, at high density on the target cell surface, is sufficient to kill M21 melanoma cells. At nonlethal C5b-8 dose, C9 caused efficient killing both at amounts insufficient for overt poly C9 formation and in C9 excess that produced abundant numbers of circular poly C9 lesions.

Methods

Human complement components. C1q (28), C8 (29), and C9 (24) were prepared as described. Purity of the isolated proteins was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using the method of Laemmli (30). Protein concentrations were determined by the Bio-Rad Laboratories (Richmond, CA) assay using bovine gamma globulin as a standard.

Radiolabeling of complement components. Purified C8 and C9 were labeled with ^{125}I using the iodogen method (Pierce Chemical Co., Rockford, IL). After labeling, iodinated protein was separated from free ^{125}I by gel filtration on a Sephadex G25 column (PD-10, Pharmacia Fine Chemicals, Piscataway, NJ). The range of specific activity was $1\text{–}2 \times 10^6$ cpm/μg. Radiolabeled C8 and C9 retained 85–100% of their hemolytic activity.

C8 and C9 depletion of human serum. Normal human serum was depleted of C8 by affinity chromatography using rabbit anti–C8 polyclonal IgG coupled to Sepharose 4B (Pharmacia Fine Chemicals). Subsequent C9 depletion was performed using affinity columns prepared from either rabbit anti–C9 polyclonal IgG or a mixture of five high-affinity murine anti–C9 monoclonal antibodies created in this laboratory. Single, double, or repeated passage over these columns yielded complete depletion as assessed by immunochemical and hemolytic criteria. Affinity chromatography was performed in the presence of 10 mM EDTA in veronal-

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1. *Abbreviations used in this paper:* C8,C9D serum, C8,C9-depleted human serum; HRF, homologous restriction factor; HSA, human serum albumin; MAb, monoclonal antibody; MAC, membrane attack complex; PI, propidium iodide; poly C9, polymerized C9.

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buffered saline with subsequent extensive dialysis of depleted serum against buffer without EDTA before storage at −70°C. The resulting C8- and C9-depleted serum (C8,9D) was reconstituted with physiologic amounts of purified C1q.

Murine monoclonal antibodies. Monoclonal antibody (MAb) 126.4, and IgM isotype with specificity for the disialoganglioside GD2, activates human complement on human melanoma cell lines expressing GD2 (31). MAb 9.2.27 (IgG2a) reacts with a melanoma associated chondroitin sulfate proteoglycan and does not activate human complement (31, 32). These MAbs were generously provided by Dr. Ralph Reisfeld from this institution.

Target cells. The M21 human melanoma cell line was originally provided by D. L. Morton (University of California, Los Angeles). MAbs 126.4 and 9.2.27 bind homogeneously by fluorescent cytometric analysis. M21 cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 20 mM L-glutamine, and gentamicin at 50 μg/ml medium. They were incubated at 37°C with 6% CO_2, and split every 2–3 d to maintain logarithmic growth. In some cultures Hyclone was replaced by 1% Nutridoma (Boerhinger Mannheim Biochemicals, Indianapolis, IN) 24 h before harvesting. After harvesting, cells were washed three times in RPMI supplemented with 1% human serum albumin (Calbiochem/Behring Diagnostics, La Jolla, CA). This medium (RPMI-HSA) was used for all experiments. Viability, determined by trypan blue exclusion, was > 90%.

Radiolabeling of target cells. 5×10^6 M21 cells in 1 ml of RPMI-HSA were labeled with either 150 μCi of ^{51}Cr or 400 μCi of neutralized ^{86}Rb (New England Nuclear, Boston, MA) for 1 h at 37°C with 6% CO_2, washed three times, and resuspended to 1×10^6/ml. Specific activities were 5,000–10,000 and 15,000–20,000 cpm/2.5×10^4 cells after ^{51}Cr and ^{86}Rb labeling, respectively.

Radioisotype release assay. Reactions were performed in duplicate in 12 × 75-mm polypropylene tubes (Costar, Cambridge, MA). Labeled M21 cells at 1×10^6/ml were incubated with an equal volume of appropriately diluted MAb 126.4 for 30 min at 37°C, 6% CO_2. C8,9D serum, reconstituted with varying doses of C8 and C9, was then added at a final dilution of 1:4 for further incubation. At various times, aliquots containing 2.5×10^4 cells were withdrawn and immediately centrifuged. 75 μl of supernatant was subjected to radioactivity measurements and percent specific release was calculated by the formula: [(experimental − spontaneous) cpm]/[maximal − spontaneous) cpm] × 100. Maximal release was determined by lysis with 0.1% NP40. Controls included C8,9D serum alone, and substitution of MAb 9.2.27 for MAb 126.4. In some experiments, where lysis was compared at 0 and 37°C, antibody-sensitized, ^{51}Cr-labeled cells were incubated with C8,9D serum for 15 min at 37°C. Aliquots were subsequently placed at 0 or 37°C, and varying amounts of C8 or C8 + C9 were added. Specific lysis was measured after 60 min at 0 or 37°C. Cells at 0° were washed at 4°C to remove unbound

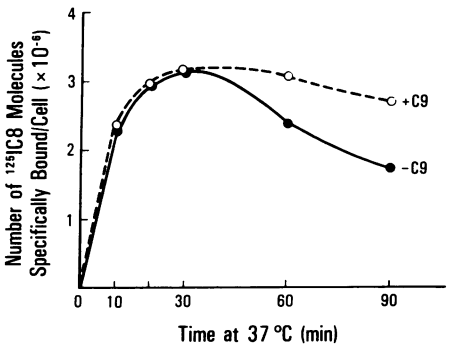

Figure 1. Specific uptake of C8 by M21 melanoma cells in the presence and absence of C9. MAb-sensitized M21 cells were incubated with C8,9D serum reconstituted with ^{125}I-C8 (60 μg/ml) in the presence and absence of C9 (60 μg/ml). At various times aliquots were processed to determine specific uptake as described in Methods. Results are the mean of three experiments with a standard deviation of < 7% for each data point.

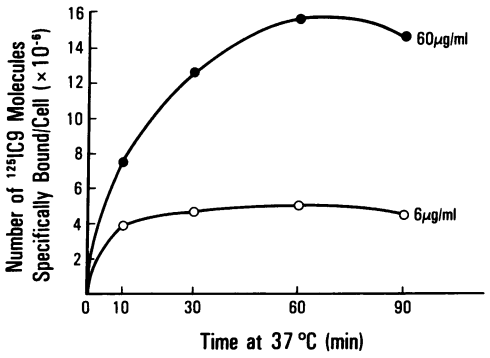

Figure 2. Specific uptake of C9 by M21 melanoma cells at different C8 concentrations. C8,9D serum was reconstituted with 6 or 60 μg/ml of C8 and ^{125}I-C9 (60 μg/ml) corresponding to C9:C8 molar ratios of 2:1 and 20:1. Nonspecific binding determined by omission of C8 was < 12% for each data point.

C8 and C9 and incubated at 37°C for 60 min, and lysis was again measured.

Deposition of ^{125}I-C8 and ^{125}I-C9 on M21 cells. Binding experiments were performed in identical fashion as radioisotope release assays, except that ^{125}I-C8 or ^{125}I-C9 was substituted for the unlabeled component. After various incubation intervals, aliquots containing 2.5×10^4 cells were layered onto 300 μl of a 1:1 mixture of dibutyl and dioctyl phthalate (Eastman Kodak Co., Rochester, NY) in 400-μl polyethylene tubes (Bio-Rad Laboratories), and immediately centrifuged for 1 min in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA). Specific binding was calculated by the formula: 100 × [(pellet) cpm]/[(pellet + supernatant) cpm] − % nonspecific binding. For C8, nonspecific binding was determined by incubation with MAb 9.2.27 instead of 126.4 or with no antibody, yielding identical results. C9-nonspecific binding was determined by omitting C8. Each time point with its corresponding controls was performed in triplicate.

Determination of propidium iodide (PI) uptake using fluorescent cytometric analysis. Incubations and dilutions were identical to radioisotope release assays. At various times, aliquots containing 3×10^5 cells were removed and immediately placed on ice. 3 ml of iced phosphate-buffered saline (PBS), pH 7.4, containing 5 μg/ml of PI (Sigma Chemical Co., St. Louis, MO) (PI-PBS) was added, and the mixture was incubated in the dark for 15 min. The cells were then washed twice with 3 ml of iced PBS, resuspended to 400 μl of PBS, and remained on ice until analysis by fluorescent cytometry. Fluorescent cytometric measurements were made in a Becton-Dickinson (Mountain View, CA) FACS IV using an argon-ion laser at 488 nm fitted with a dichroic mirror (570 DM), and a 625/35-nm band pass filter to detect PI (FL2).

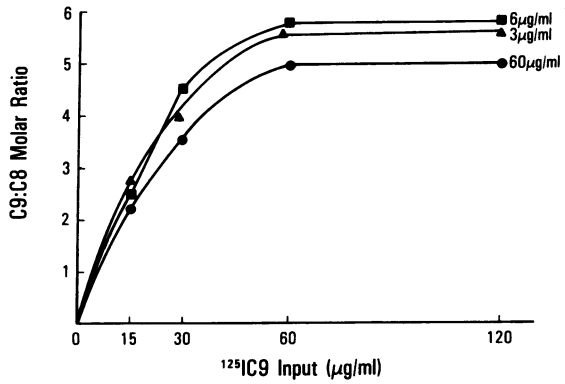

Figure 3. Molar ratio of C9:C8 bound to M21 melanoma cells at different C8 concentrations (3, 6, and 60 μg/ml). The C9:C8 ratio was calculated from binding studies as described in Figs. 1 and 2.

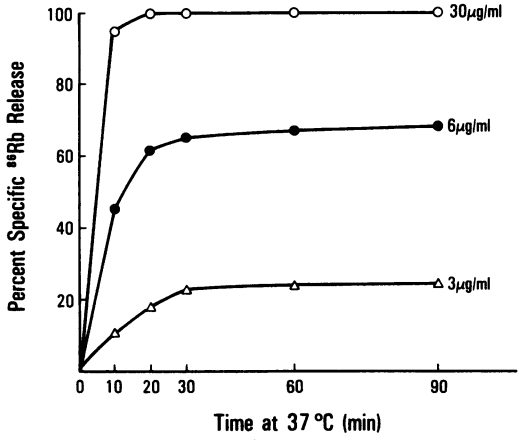

Figure 4. Kinetics of ^{86}Rb release from M21 melanoma cells at different C8 concentrations (3, 6, and 30 μg/ml) in the absence of C9. Maximal release was determined using 0.1% NP-40. Spontaneous release was 15% at 60 min. Each data point was determined in duplicate.

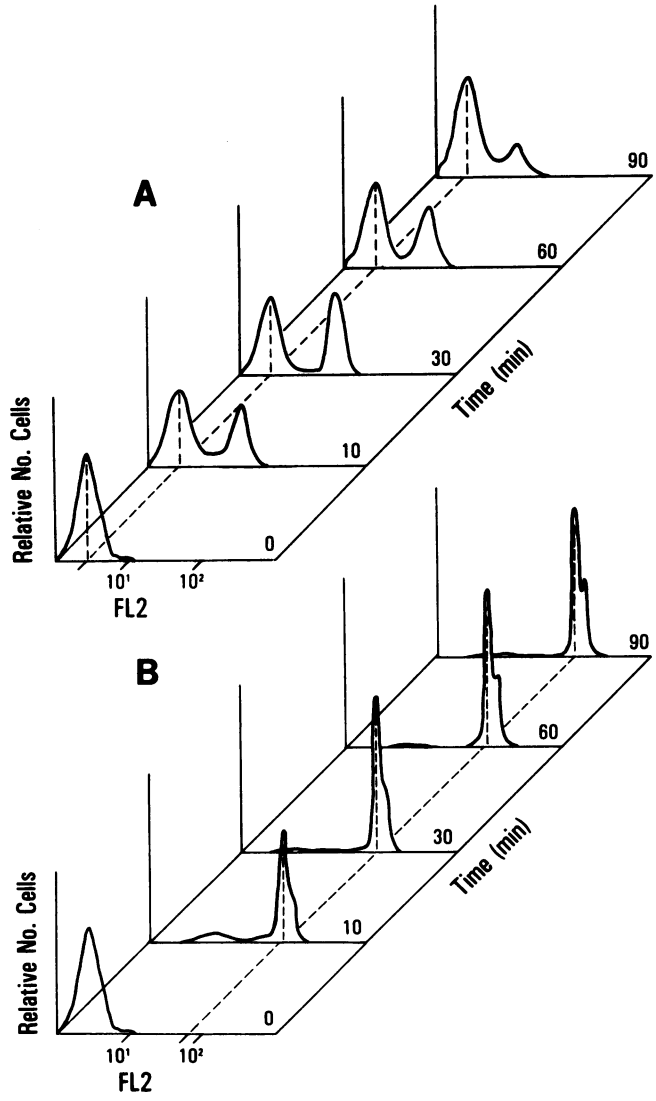

Figure 5. Fluorescent cytometric analysis of PI (FL2) uptake by M21 cells. Cells were incubated at 37°C with C8,9D serum reconstituted with C8 (6 μg/ml) (*A*) in the absence of C9 and (*B*) with C9 (60 μg/ml). At various times, aliquots were placed on ice and treated with PI-PBS as outlined in Methods.

Electron microscopy. Incubations and dilutions were as described above for kinetic assays. For transmission electron microscopy, 2×10^4 cells were washed twice in PBS and resuspended in 1 ml of 5 mM Na-Hepes, pH 7.2, to disrupt the cell. A 10-μl sample was applied to a carbon-coated grid, 400 mesh, and left at room temperature for 30 min to dry. Negative staining was then performed with 1% uranyl formate for 30 s with excess removed by blotting on filter paper. The grid was examined with a Hitachi 12A transmission electron microscope (Tokyo, Japan) using an acceleration voltage of 75 kV and magnification of 30,000 or 60,000. For scanning electron microscopy, similar samples were fixed in modified Karnovsky's fixative (1.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) at 4°C for 1 h, followed by rinsing and deposition onto glass slides precoated with poly-L-lysine. Samples were dried, carbon coated, and observed with a Hitachi S 500 scanning electron microscope operating at 20 kV accelerating voltage.

Analysis of MAC-associated poly C9 by SDS polyacrylamide gradient gel electrophoresis. 1×10^5 M21 cells in 0.1 ml of RPMI-HSA were treated as in previous experiments with reconstitution of C8,9D serum with C8 and ^{125}I-C9. After 60 min of incubation at 37°C, 6% CO_2, the cells were washed once with PBS and lysed with H_2O. After centrifugation, sediments were dissolved in 70 μl of 4% SDS with 1% β-mercaptoethanol, boiled for 15–20 min, and subjected to electrophoresis on 2.5–10% polyacrylamide gradient slab gels utilizing the Laemmli system. Controls on every gel included tubular poly C9 generated by addition of 50 μM Zn^{2+} and prolonged incubation at 37°C, monomeric C9, and ^{125}I-C9. Gels were stained with Coomassie Brilliant Blue and sliced in 0.5–1-cm sections, and radioactivity was measured to determine the fraction of bound C9 converted to SDS-resistant poly C9. > 1% of the ^{125}I-C9 control migrated as the 1.1×10^6 mol wt SDS-resistant poly C9.

Results

Kinetic analysis of radiolabeled C8 and C9 uptake by M21 melanoma cells. Fig. 1 shows a kinetic analysis of binding of ^{125}I-C8 (60 μg/ml) in the presence and absence of unlabeled C9. Uptake curves were identical during the first 30 min when maximal binding occurred. The discrepancy between the two curves at times > 30 min is probably attributable to the slower kinetics of killing in the absence of C9 allowing active shedding of C5b-8 complexes. Specific ^{125}I-C9 binding was saturable at physiologic concentrations (60 μg/ml), independent of C8 concentration, and was not enhanced by five times physiologic doses of C9 (not shown). Fig. 2 is a kinetic analysis of C9 uptake at saturating

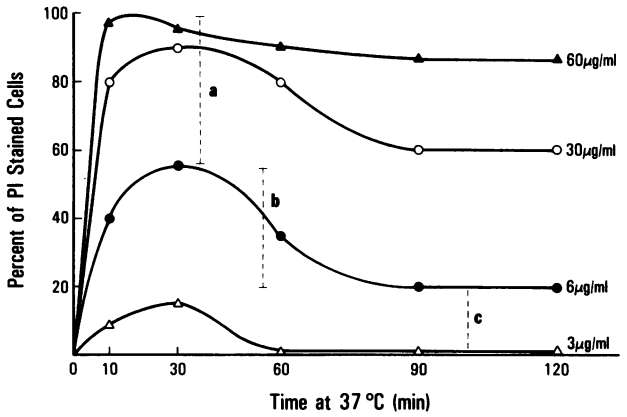

Figure 6. Kinetics of PI uptake by M21 cells at different C8 concentrations (3, 6, 30, and 60 μg/ml) in the absence of C9. PI uptake profiles, derived from serial fluorescence histogram analyses as in Fig. 5, reveal three subpopulations of cells indicated in the figure for the 6-μg/ml C8 dose: (*a*) resistant, (*b*) reversibly damaged, (*c*) irreversibly damaged.

*Table I. Recovery of M21 Melanoma Cells Attacked by C5b-8 or C5b-9 (at Nonlytic C8 Concentration) as Measured by Propidium Iodide Uptake**

C8 Concn.	C9 Concn.	Percent cells with PI uptake		Percent recovery
		30 min	90 min	
μg/ml	μg/ml	%	%	
3	0	15	0	100
6	0	55	20	64
30	0	90	60	33
3	1.5	45	20	55
3	3	60	35	38
3	6	90	72	20
3	60	97	92	5

* M21 cells (1×10^6 cells/ml) were incubated with C8,9D serum containing various doses of C8 or C8 and C9. At indicated times, aliquots containing 3×10^5 cells were removed and briefly incubated with propidium iodide as described in Methods. Percent recovery is derived from the ratio of PI-positive cells at 90 and 30 min. Results are the mean of three experiments with standard deviations of < 10% of each number.

doses with C8 concentrations corresponding to C9:C8 molar ratios of 2:1 and 20:1. The molar ratio of bound C9:C8 was determined as a function of C9 concentration (Fig. 3) at various C8 concentrations. The C9:C8 ratio was maximal at physiologic concentrations of C9 with the plateau at 60 min as predicted from the delayed C9 binding relative to C8 (Figs. 1 and 2).

Formation and elimination of C5b-8 and C5b-9 channels in M21 melanoma cells. The kinetics of intracellular ^{86}Rb release as a function of C8 concentration in absence of C9 is shown in Fig. 4. Rapid total release was observed with C8 at high concentrations (30 μg/ml). Rapid, but submaximal, transmembrane ion flux was seen at decreasing doses of C8 (6 and 3 μg/ml) with plateauing at ~ 30 min of incubation, corresponding to maximal C8 binding.

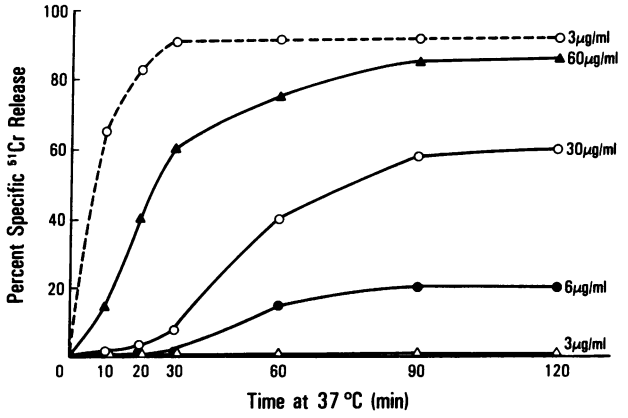

Figure 7. Killing of M21 melanoma cells in the absence of C9 at different C8 concentrations (3, 6, 30, and 60 μg/ml). Kinetics of specific ^{51}Cr release were determined as outlined in Methods. *Dashed line* represents lysis due to addition of C9 (60 μg/ml) to a nonlytic dose of C8 (3 μg/ml). Results are the mean of three experiments with a standard deviation of < 5% for each data point. Spontaneous ^{51}Cr release was 7–10% at 120 min.

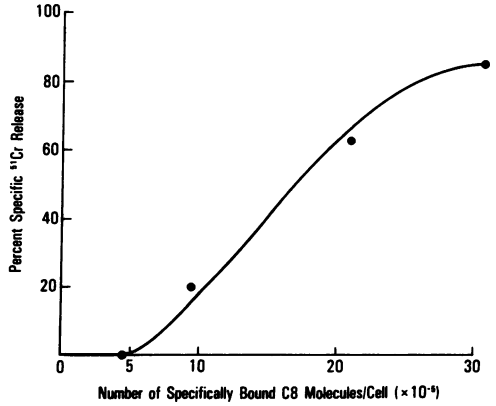

Figure 8. Killing of M21 melanoma cells by cell-bound C5b-8 in the absence of C9. ^{51}Cr release and ^{125}I-C8 uptake were determined in parallel experiments. Incubation time was 30 min for ^{125}I-C8 and 90 min for ^{51}Cr release. Spontaneous ^{51}Cr release was 5–9% at 90 min.

The second method of measuring loss of membrane integrity as a consequence of functional complement channel formation was the uptake of PI. Fig. 5 *A* shows serial histograms obtained by fluorescent cytometric analysis of PI uptake as a function of time at a C8 concentration of 6 μg/ml in the absence of C9. A shift is observed in the distribution of cells capable of excluding PI, with stabilization at 90 min. Fig. 5 *B* shows the results of adding a physiologic amount of C9 (60 μg/ml). In comparing the positively fluorescent cells in Fig. 5, *A* and *B*, it can be seen that there are two different intensities. The positively stained cells in Fig. 5 *A* have a diffuse cytoplasmic staining pattern by fluorescence microscopy, reflecting loss of plasma membrane integrity. Positively stained cells in Fig. 5 *B* have, in addition, very brightly stained nuclei, reflecting loss of integrity of both plasma and nuclear membranes. These observations are consistent with the 100-fold increase in quantum fluorescence efficiency of PI upon intercalation with DNA.

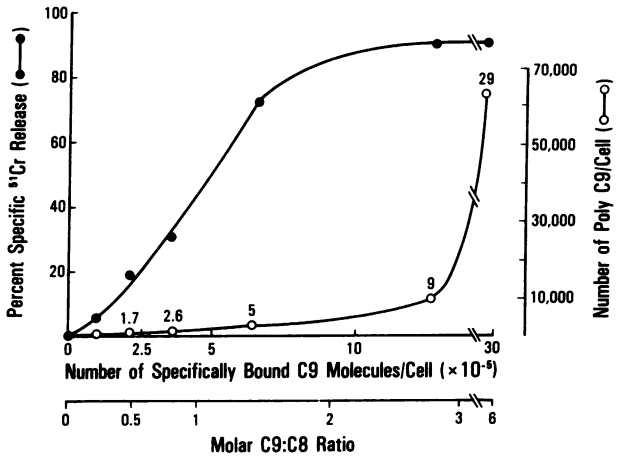

Figure 9. Killing of M21 melanoma cells in relation to number of specifically bound C9 molecules per cell and to the molar C9:C8 ratio. Cells were incubated with C8,9D serum, reconstituted with a nonlytic dose of C8 (3 μg/ml) and varying doses of C9 for 90 min at 37°C. Numbers above open circles indicate percentage of cell-bound C9 molecules participating in SDS-resistant poly C9. Spontaneous ^{51}Cr release was 5–9% at 90 min.

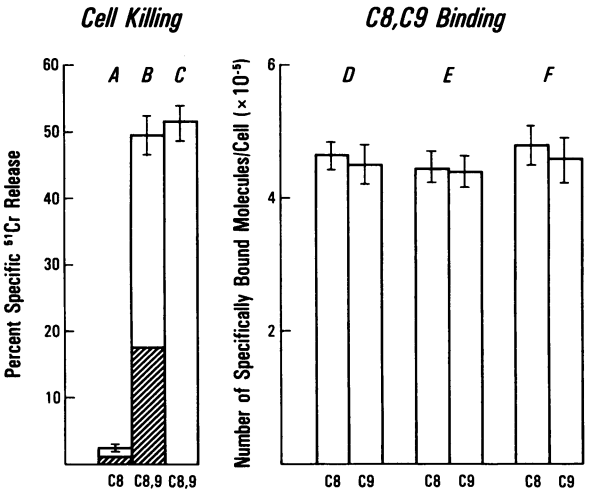

Figure 10. Killing of M21 melanoma cells by C5b-$8_1 9_1$ at 0°C and 37°C. ^{51}Cr-labeled cells were incubated with C8,9D serum for 15 min at 37°C. (*A, B*) Cells were then placed at 0°C and C8 (3 μg/ml) or C8 + C9 (3 μg/ml) were added with incubation for 60 min, when specific lysis was measured (shaded columns). Aliquots were subsequently washed at 4°C to remove free C8 and C9 and lysis was measured after incubation at 37°C for 60 min (unshaded columns). (*C*) Cells were incubated only at 37°C for 60 min. Spontaneous ^{51}Cr release was 6–9%. Under identical conditions, unlabeled cells were incubated with ^{125}I-C8 or C9 as described in methods. (*D*) C8,C9 binding to cells incubated for 60 min at 0°C. (*E*) Aliquots washed at 4°C and incubated for 60 min at 37°C. (*F*) Cells incubated for 60 min at 37°C only. C9:C8 ratio was ~ 1:1. Error bars indicate the mean of three measurements ± standard error of the mean.

Fig. 6 graphically depicts the kinetics of PI uptake at various C8 concentrations without C9. Analysis of C5b-8 mediated PI uptake profiles reveals three subpopulations of cells: (*a*) resistant, with no PI uptake; (*b*) reversibly damaged, regaining the ability to exclude PI with further incubation; and (*c*) irreversibly damaged, with a stable PI uptake at 90 min. These subpopulations are indicated in Fig. 6 for the 6 μg/ml dose of C8. Table I shows the relative recovery of M21 cells ranging 14–100% at various C8 doses in absence of C9. Addition of various doses of C9 to nonlytic amounts of C5b-8 markedly reduced the number of cells capable of eliminating complement channels (5–55%).

Killing of M21 melanoma cells by C5b-8. Fig. 7 shows the kinetics of ^{51}Cr release at different C8 doses without C9. C5b-8 mediated lysis was maximal at 90 min, and the final extent of the reaction was markedly sensitive to C8 concentration. At a C8 input of 60 μg/ml, lysis approached 85% and 3.1×10^6 C8 molecules were specifically bound per cell (Fig. 8). At 6 μg of input, lysis was 20% and 900,000 C8 molecules were bound. At the lowest input (3 μg/ml), 470,000 C8 molecules were still bound per cell, but no killing was observed although membrane leakiness was detectable by ^{86}Rb release (Fig. 4) and PI uptake (Fig. 6). With addition of physiologic amounts of C9 to the nonlytic amount of C8 (3 μg/ml) lysis was rapid, reaching maximal ^{51}Cr release at 30 min. The shape of the kinetic curves of C8-dependent lysis (Fig. 7) and the sigmoidal dose response curve (Fig. 8) suggest cooperativity of C8 sites on the target cell to achieve effective membrane lesions. This is also suggested by the high number of C8 sites required for lysis in absence of C9.

Killing of M21 melanoma cells in relation to the molar ratio of cell bound C9:C8 and poly C9 formation. Fig. 9 depicts C9 lytic efficiency as a function of the number of specifically bound C9 molecules and the C9:C8 molar ratio at a nonlytic dose of C8 (3 μg/ml). Also indicated is the extent of poly C9 formation under these conditions. Maximal killing was achieved at a C9:C8 ratio of 3:1, with < 10% of bound C9 converted to poly C9. At a molar C9:C8 ratio of 1:1, killing was ~ 50% and of the 470,000 C9 molecules bound per cell, ~ 12,000 participated in the formation of the 1,000 SDS-resistant poly C9 structures detected. To further evaluate the contribution of these poly C9 structures to cell lysis, ^{51}Cr-release and C8,C9-binding measurements were performed at 0°C (Fig. 10). Under these conditions, C5b-7–treated cells bind C8 and C9 to form C5b-$8_1 9_1$ (33). Cells incubated at 0°C for 60 min showed 15–20% lysis at a C9:C8 molar ratio of ~ 1:1 (Fig. 10, *B* and *D*). After washing, further incubation at 37°C for 60 min yielded a total lysis of 50% with no change in the C9:C8 ratio (Fig. 10, *B* and *E*). There was no significant difference in lysis or C9:C8 ratio when cells were incubated for 60 min at 37°C only (Fig. 10, *C* and *F*). These results cumulatively provide evidence for a minimal contribution of circular poly C9 to the lytic efficiency of MAC at low C9:C8 molar ratios. Table II summarizes the relation of specific C8 and C9 binding to formation of poly C9 at various C9:C8 molar ratios.

Electron microscopic studies of M21 melanoma cells killed by either C5b-8 or C5b-9. Cells bearing various numbers of C5b-8 or C5b-9 complexes were examined by transmission electron microscopy. Membranes of cells incubated with C8,9D serum and physiologic concentrations of C8 and C9 revealed a high density of the classical circular MAC lesions (Fig. 11 *A*). Patching of lesions seen in most fields was attributed to antibody cross-linking of the mobile GD2 antigen as visualized by fluorescence

Table II. C9:C8 Molar Ratios and Poly C9 Formation as a Function of C8 and C9 Uptake

C8 molecules per cell	C9 molecules per cell	C9:C8 ratio	Poly C9 per cell*	Poly C9
				%
3.1×10^6 (60)‡	1.6×10^7 (60)	4.9	6.8×10^5	51
3.1×10^6	1.1×10^7 (30)	3.5	2.5×10^5	28
3.1×10^6	6.3×10^6 (15)	2.1	5.0×10^4	9
3.1×10^6	3.3×10^6 (6)	1.1	2.2×10^4	8
9.0×10^5 (6)	5.7×10^6 (60)	5.9	1.8×10^5	37
9.0×10^5	4.2×10^6 (30)	4.6	7.3×10^4	21
9.0×10^5	2.5×10^6 (15)	2.5	1.4×10^4	7
9.0×10^5	1.2×10^6 (6)	1.3	5.0×10^3	5
4.7×10^5 (3)	2.7×10^6 (60)	5.7	6.4×10^4	29
4.7×10^5	1.8×10^6 (30)	3.9	2.5×10^4	17
4.7×10^5	1.2×10^6 (15)	2.8	9.7×10^3	9
4.7×10^5	6.6×10^5 (6)	1.4	2.7×10^3	5
4.7×10^5	3.7×10^5 (3)	0.78	8.0×10^2	2.6
4.7×10^5	2.1×10^5 (1.5)	0.44	2.9×10^2	1.7
5.5×10^4 (0.3)	3.6×10^5 (60)	6.6	ND	ND
3.4×10^4 (0.15)	2.2×10^5 (60)	6.5	ND	ND

*Calculated assuming 12 C9 monomers per SDS-resistant poly C9.
‡C8,C9 binding and poly C9 formation was determined after 60 min of incubation with C8,9D serum with numbers in parentheses indicating C8,C9 input in μg/ml.

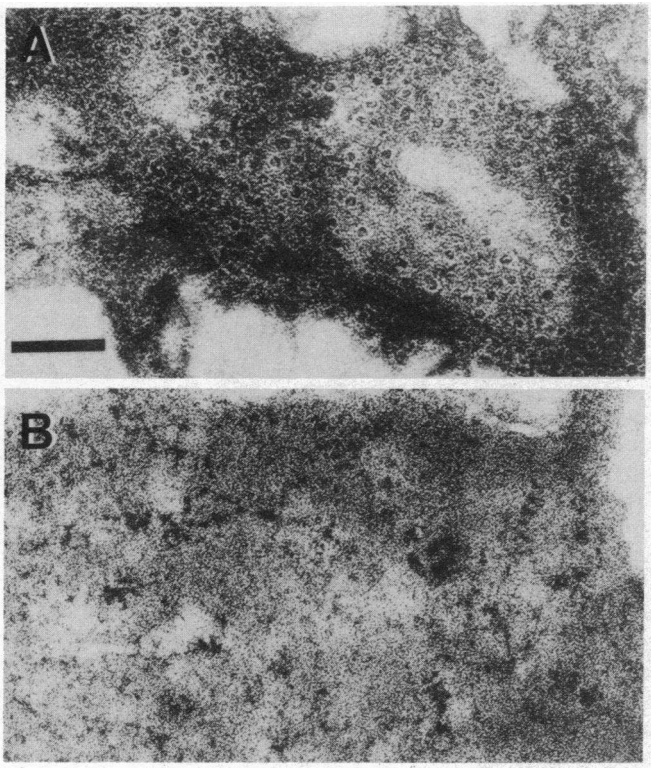

Figure 11. Transmission electron microscopy of M21 melanoma cell membranes. Cells attacked by (*A*) C5b-9 resulting in the binding of $\sim 3 \times 10^6$ C8 molecules per cell and 16×10^6 C9 molecules per cell and (*B*) C5b-8 resulting in the binding of 3×10^6 C8 molecules/cell. Bar, 100 nm.

microscopy. Circular lesions were not detectable at C9:C8 ratios of less than 3:1, regardless of C5b-8 density. Cells bearing up to 3×10^6 C5b-8 complexes showed no circular membrane lesions in absence of C9 (Fig. 11 *B*).

Scanning electron microscopy revealed qualitative differences between cells lysed by C5b-8 and those lysed by C5b-9. M21 melanoma cells attacked by C8,9D serum and physiologic doses of C8 (60 μg/ml) in absence of C9 showed membrane blebbing at 30 min (Fig. 12 *B*), at which time all cells had released ^{86}Rb and taken up PI and ~ 50% had died. At 90 min 90% of the cells had died and the cell depicted in Fig. 12 *C* shows vesiculation and appears collapsed. In Fig. 12 *D* an M21 cell attacked by C5b-9 exhibits disintegration of its membrane structure at 30 min. The appearance of a cell treated with C8,9D serum without further additions (Fig. 12 *A*) is similar to that of untreated cells.

Discussion

This work was conducted to determine the molecular conditions that lead to complement-mediated killing of nucleated cells with respect to C8, C9, and poly C9. The information obtained may aid in the design of immunotherapy utilizing host complement (34). It also may serve in comparing the cytotoxic efficiency of the C9-related protein of killing lymphocytes (35, 36) with that of C8 and C9.

The main questions addressed were (*a*) whether tubular poly C9 was needed for the killing of nucleated human cells; (*b*) how these cells respond to massive C5b-8 attack; and (*c*) which conditions of complement attack allow membrane repair and cell recovery.

Initial experiments were directed toward determining the molar C9:C8 ratio at saturation of C9 binding varying cell-bound C8 between 34,000 and 3.1 million molecules per cell. The maximal ratio achieved over this broad range was 6:1 (Fig. 3, Table II). This is an average value representing MAC structures presumably containing 0–12 or more molecules of C9. In fact, at the highest numbers of cell-bound C8 and C9 molecules employed (3.1×10^6 and 1.6×10^7, respectively), the yield of poly C9 was 51% (Table II).

The experiments with C5b-8 in absence of C9 showed that membrane damage caused by C5b-8 is, in part, reversible and that C5b-8, at high multiplicity, is capable of killing human melanoma cells. The methodology of this study allowed correlation between the number of molecules specifically bound to the target cell and functional channel formation. Membrane leakiness due to C5b-8 channels was detected by ^{86}Rb release (Fig. 4) or propidium iodide uptake (Fig. 6). ^{86}Rb release was dose dependent and rapid. It was complete at 30 min at which time PI uptake was maximal. Membrane permeability for PI markedly decreased thereafter and reached a constant level at 90 min of incubation, indicating membrane repair or channel elimination. Recovery by 90 min was between 55% and 64% under conditions at which 45–55% of cells were PI permeable at 30 min (Table I).

An excellent correlation was found between PI uptake and cell death as measured by ^{51}Cr release after 90 min of C5b-8 attack (Figs. 6 and 7). This agrees with previous comparisons of these cell "viability" techniques (37). No such correlation existed at 30 min of incubation. At a C8 input of 3–30 μg/ml, PI uptake was 15–90%, but there was little or no cell killing. Maximal killing (85–90%) by C5b-8 required more than 3 million specifically bound C8 molecules per cell. C5b-8 has been shown to bind phospholipid (38) and weaken membrane structure (39). Its membranolytic activity has been described in erythrocytes (24, 25), *Neisseria gonorrhoeae* (40) and more recently in *Giardia lamblia* (27) and the human cell line U937 (26). However, *Escherichia coli* and various cell lines have been reported to be resistant to C5b-8 (4, 10, 41). Resistance of nucleated cells to lysis by C5b-8 may be a relative phenomenon and directly related to the number of attacking C5b-8 complexes and possibly to the rate of attack. The binding curve for radiolabeled C8 (Fig. 1), representing the net effect of uptake and elimination, indicates that a rapid, overwhelming attack by C5b-8 complexes is required for ultimate lysis. The M21 melanoma cell line is highly resistant to C5b-8 lysis; as many as 470,000 complexes bound per cell did not produce any detectable cell killing (Fig. 8). In contrast, in a recent study by Morgan et al. (26), U937 cells were effectively lysed by 80,000 C5b-8 complexes per cell. Homologous restriction factor (HRF), a membrane protein that interacts with C8 and C9 during MAC assembly and inhibits channel formation (42), was hypothesized to be at least partially responsible for the large number of C5b-8 complexes required for M21 cell killing. However, immunoblot and FACS analyses failed to detect HRF on M21 cells. Furthermore, antiserum to HRF was unable to enhance reactive lysis of these cells (manuscript in preparation).

Two distinct C5b-9 cytolytic mechanisms may be distinguished. Using a noncytolytic amount of C5b-8 and limiting quantities of C9, the molar ratio of cell-bound C9:C8 was below 3:1 and tubular poly C9 formation was < 10% of total C9 bound (Table II). Under these conditions no circular MAC images could be visualized by electron microscopic inspection of the target

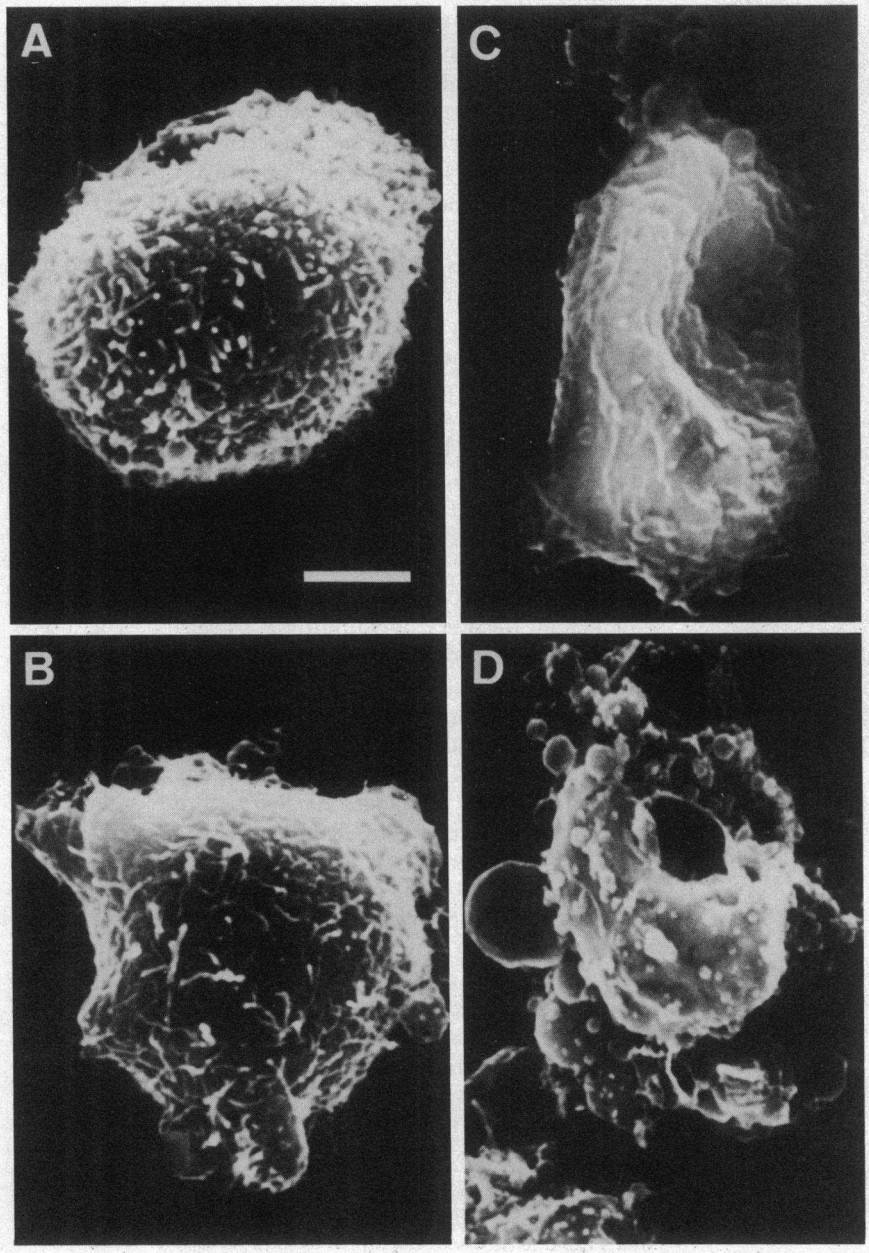

Figure 12. Scanning electron microscopy of M21 melanoma cells attacked by C5b-8 and C5b-9. Cells were incubated with (*A*) C8,9D serum for 90 min, (*B, C*) C8,9D serum reconstituted with 60 μg/ml of C8 for 30 and 90 min, respectively, (*D*) C8,9D serum plus C8 and C9 (60 μg/ml) for 30 min. Bar, 4 μm.

membranes (like Fig. 11 *B*). However, cell killing approached 100% (Fig. 9). This mechanism is consistent with the formation of large C5b-9 aggregates in the target membrane that produce channels of various sizes and exhibit membranolytic activity. When the MAC was formed at 0°C, consisting primarily of C5b-$8_1 9_1$ (33), and the cells were washed before they were incubated at 37°C, cell killing was similar to that observed when circular poly C9 formation was allowed to occur (Fig. 10). Thus, at low C9:C8 molar ratios, where circular poly C9 formation is minimized or prevented, lytic efficiency of the MAC is not impaired.

The second mechanism involves the supply of an excess of C9 which results in molar ratios of cell-bound C9:C8 approaching 6:1. Under these conditions 30 to 50% of the bound C9 had formed SDS-resistant poly C9 (Table II) and clusters of negatively staining circular lesions were readily observed by electron microscopy on the membranes of the killed cells (Fig. 11 *A*). Both mechanisms exhibit cytotoxic efficiency in the killing of human melanoma cells.

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