ENHANCED REACTIVE LYSIS OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA ERYTHROCYTES

Studies on C9 Binding and Incorporation into High Molecular Weight Complexes

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The human disease paroxysmal nocturnal hemoglobinuria (PNH)[1] may be regarded as an experiment of nature that has already provided, and should continue to provide, unique insights on the cytolytic function of complement (C) and its regulation (1–4). The studies of our laboratory have been focused particularly on elucidating the process by which the most sensitive type of PNH erythrocyte (E) (designated type III) are abnormally sensitive to in vitro reactive lysis, i.e., to membrane attack initiated by purified C5b6, C7, C8, and C9 in the absence of the earlier activation and amplification steps (5).

In an earlier report (6), we presented evidence that for a given input of purified C5b6, type III PNH E (PNH-III E) did not bind ^{125}I-C7 to a greater extent than normal human E. Rather, the data indicated that PNH E underwent two- fourfold greater lysis for a given number of C7 molecules bound. Compatible results were obtained in less extensive, unpublished studies with radiolabeled C5b6. Compelling independent evidence from other laboratories (7) indicates that the basic unit of the membrane attack complex involves one molecule each of C5b, C6, C7, and C8 in the formation of the C5b-8 complex, to which a variable number of C9 molecules then bind to produce the fully effective membrane attack complexes. Therefore, although C8 uptake has not been directly measured in our system, it seems justified to infer that in reactive lysis the assembly of C5b-8 complexes on PNH-III E from the fluid phase is quantitatively normal. The excessive lysis of the PNH E by the full C5b-9 sequence could result from a quantitative abnormality affecting C9 binding and/or polymerization within the C5b-9 complex, or from a qualitative difference in the disposition or function of the terminal C components after they have become bound to the PNH membrane. The previously reported deficiency of a membrane complement regula-

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[1] *Abbreviations used in this paper:* DAF, decay-accelerating factor; EA, antibody-sensitized E; $GVBE_{6.5}$, isotonic barbital buffer, pH 6.5, with EDTA and gelatin; HMW, high molecular weight; $HSA\text{-}VBE_{7.4}$, isotonic barbital buffer, pH 7.4, with human serum albumin and EDTA; MAC, membrane attack complexes; PNH, paroxysmal nocturnal hemoglobinuria; $SGVB^{2+}$, half-isotonic barbital buffer, pH 7.4, with sucrose, Ca^{2+}, and Mg^{2+}.

tory protein, decay-accelerating factor (DAF), in type II and type III PNH E (3, 4) accounts for increased C3b binding to these cells during classical or alternative pathway activation in whole serum, but does not appear to be involved in the regulation of the susceptibility of E to reactive lysis (8, 9).

In this report we compare the binding of ^{125}I-C9 to type II and III PNH E and normal E in reactive lysis, as well as in classical pathway-initiated lysis, with attention to both total specific binding and the proportion of cell-bound C9 in high molecular weight (HMW) forms. A preliminary account of these findings has been presented (10).

Materials and Methods

Cells. Erythrocytes from two PNH patients with >95% type III E, one patient with >90% type II E, and from normal controls, were obtained and stored frozen at −80°C as described previously (5). PNH and normal E frozen for approximately equal times were thawed on the same day, washed, and stored in Alsever's solution at 2°C until used simultaneously in experiments (generally <2 wk after thawing).

Complement Components. Human C5b6 was purified by a modification of the method of Yamamoto and Gewurz (11) in which a C5/C6 reagent depleted of C7 was prepared by pooling the C5- and C6-containing fractions from a DEAE Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) column as described by Hammer et al. (12). Cleavage of C5 using serum-treated zymosan and subsequent purification of C5b6 were as reported (11). Human C7 was prepared and radiolabeled as previously described (6). C9 was purified by a combination of published methods. The C9-containing fractions from a DEAE Sephacel column (12) were concentrated and applied to a 2.5 × 100 cm column of Sepharose 6B (Pharmacia Fine Chemicals), which had been equilibrated in 25 mM barbital buffer, pH 7.0, containing 100 mM NaCl and 0.5 mM PMSF (13). The column was eluted with starting buffer and the C9-containing fractions were pooled and applied to a 2.6 × 4.5 cm column of hydroxyl apatite (HA Ultrogel; LKB Instruments, Inc. Gaithersburg, MD), which had been equilibrated in the buffer used for the preceding column. The column was washed with starting buffer and then with 80 mM Na^+/K^+ phosphate buffer, pH 7.7, after which a 9-column volume linear gradient of 80–400 mM Na^+/K^+ phosphate buffer, pH 7.7, was applied. The C9-containing fractions were pooled and concentrated using a PM30 membrane (Amicon Corp., Danvers, MA). Guinea pig C1 (14) and human C3, C6, C8 (12), and C5 (15) were purified by published methods. Human C2 and C4 were purchased from Cordis Laboratories Inc. (Miami, FL), and C2 was oxidized with I_2 (16) just before use.

Radiolabeling. The C9 preparations were radiolabeled with ^{125}I using the Iodogen method (Pierce Chemical Co., Rockford, IL) at 0°C, according to the manufacturer's instructions. After decanting from the Iodogen-coated tubes and quenching with cold NaI, we added 1 mg/ml of human serum albumin (Sigma Chemical Co., St. Louis, MO). Unbound ^{125}I was removed by overnight dialysis at 4°C against phosphate buffered saline, pH 7.5, containing 0.01 M EDTA, with Rexyn 201 beads (Fisher Scientific Co., Pittsburgh, PA) added to the chamber. The final preparations containing 25 μg of radiolabeled C9 were absorbed twice at 0°C with 1.25×10^9 washed normal human E. This step removed ~18% of the radioactivity, and it reduced nonspecific C9 binding without significantly changing the C9 hemolytic titer of the radiolabeled preparation. The final preparations used for the binding experiments reported here contained 1.55–1.96 μCi/μg C9 and the radioactivity was 92–99% precipitated by 10% TCA. Some experiments were performed with C9 radiolabeled by the lactoperoxidase method (Enzymobeads; Bio-Rad Laboratories, Rockville Center, NY). These preparations did not require absorption with normal E. Results using C9 radiolabeled by either method were comparable. The C9 concentration of each radiolabeled preparation was measured by electroimmunoassay using specific anti-C9 (Miles Laboratories, Elkhart, IN). The reference standard for C9 was a pool of 14 normal human sera, which was assumed to contain 58 μg C9/ml (13).

The C9 preparations appeared homogeneous by Coomassie blue staining of SDS-PAGE gradient gels. Using radiolabeled preparations, autoradiography of such gradient gels with or without reducing conditions revealed that 91–97% of the radioactivity appeared in the monomeric C9 band. For some experiments, the radiolabeled C9 was applied to a column of Sephadex G-200 (Pharmacia Fine Chemicals) to remove any SDS-sensitive C9 aggregates formed during the radiolabeling procedure.

C9 Binding. Frozen E of PNH-III, PNH-II, and normal individuals were thawed on the same day, washed in 5 mM barbital buffer, pH 6.5, containing 150 mM NaCl, 20 mM EDTA and 0.1% gelatin ($GVBE_{6.5}$), and were standardized by electronic counting (Coulter Electronics Inc., Hialeah, FL). 3.5×10^8 E of each type were pelleted and the supernatant was removed. Excess C5b6 in $GVBE_{6.5}$ was placed in each tube and the tubes were incubated at 37°C for 10 min in a shaking water bath. Then, 230 ng C7 in the same buffer were added (final volume, 140 μl), and the tubes were incubated another 10 min at 37°C. The resulting EC5b67 were washed twice in 5 mM barbital buffer, pH 7.4, containing 150 mM NaCl, 20 mM EDTA, and 0.3% human serum albumin ($HSA\text{-}VBE_{7.4}$), were resuspended with 3.8 ml of the same buffer, and aliquots of these suspensions were removed for cell counts. In a typical experiment, 3×10^7 EC5b67 of each cell type in 325 μl HSA-VBE were placed in a series of 11 × 75–mm polycarbonate tubes. Duplicate sets of tubes for each cell type received either of two dilutions of C8 (25 μl), buffer ($HSA\text{-}VBE_{7.4}$, 25 μl), or NH_4OH (to determine 100% lysis). 34 ng of radiolabeled C9 in 25 μl $HSA\text{-}VBE_{7.4}$ were added to each tube, and incubation proceeded for 60 min at 37°C; then 2.5 ml of cold saline containing 0.09 M EDTA (pH 7.4) was added to each tube. Unlysed cells and ghosts were pelleted by centrifugation at 17,000 rpm for 10 min at 4°C in a Sorvall RC-2B centrifuge with an SM24 head (Dupont Co., Wilmington, DE). The supernatant was removed and released hemoglobin was determined by absorbance at 413 nm for the calculation of lysis. The pellet was washed sequentially in 60, 30, and 20 mosmol phosphate buffers, pH 7.5, with the ghosts recovered at each step by centrifugation at 17,000 rpm for 10 min at 4°C. After the final 20 mosmol wash and removal of the supernatant, the residual white ghosts were transferred quantitatively to fresh polycarbonate tubes by using four 0.5-ml aliquots of 20 mosmol buffer, and were recentrifuged. The supernatant was then removed with a measured aspirator leaving 100 μl in each tube. 100 μl of double-strength SDS-PAGE sample buffer (see below) were added to each tube and the tubes were placed in a boiling water bath for 1.5 min. Two aliquots of 90 μl each were removed from each tube and counted for radioactivity; nonspecific counts bound in the absence of C8 were subtracted from the total to obtain specific binding. The molecules of C9 bound were calculated using the measured specific activity of the C9 preparation, assuming a molecular weight for C9 of 71,000 (13). Actual cell counts of the EC5b67 suspension were used to calculate molecules of C9 bound per E.

In some experiments we used different cell numbers and volumes than those described above. Other modifications included substituting 0.1 or 0.2% gelatin for 0.3% HSA in the buffer used in the C8 and C9 steps; substituting 5 mM Mg^{2+} and 1.5 mM Ca^{2+} for the EDTA in this buffer; and performing the entire reactive lysis procedure in a single step, wherein C5b6 was added to tubes containing E, C7, C8, and radiolabeled C9, either in EDTA or in Ca^{2+}- and Mg^{2+}-containing buffers. Results with these modifications were comparable to those obtained using the basic method described above.

C9 Binding During Classical Pathway-initiated Lysis. PNH and normal E (1.2×10^8 cells/ml) were suspended in 2.5 mM barbital buffer, pH 7.4, containing 75 mM NaCl, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1% gelatin, and 0.14 M sucrose ($SGVB^{2+}$), and were incubated for 20 min at 37°C with an equal volume of $SGVB^{2+}$ containing appropriate dilutions of heat-inactivated (56°C for 60 min) alloimmune human anti-Tj^a (5), guinea pig Cl, and human C4. These EAC14 were washed in cold $SGVB^{2+}$, resuspended to 1.5×10^8 cells/ml, and incubated with excess C2 oxy, C3, C5, C6, and C7 in the same buffer for 30 min at 37°. The resulting EAC1-7 were washed in $HSA\text{-}VBE_{7.4}$ and treated with C8 and ^{125}I-C9 as described above for EC5b-7 generated by reactive lysis. In some experiments, washed EAC14 were incubated with a mixture of purified C2, C3, C5, C6,

C7, C8, and varying doses of ^{125}I-C9 in $SGVB^{2+}$ for 60 min at 37°C, and then were treated as above to determine lysis and C9 binding.

Polyacrylamide Gel Electrophoresis. Vertical slab PAGE was performed according to the method of Laemmli (17) by using an SE600 apparatus (Hoefer Scientific Instruments, San Francisco, CA). Linear gradients of 2.3–11% polyacrylamide were poured using Gel Bond-Pag supports (FMC Corp., Rockland, ME). Running buffers contained 0.1% SDS (Bio-Rad Laboratories), and the sample buffers in which the ghosts were dissolved contained either 0.1 or 2% SDS (final concentrations); 80 μl of each sample were applied to the gel after radioactivity was counted. In some experiments we used 0.5% SDS in the sample buffer. Since 0.1% SDS was the lowest concentration that consistently fully solubilized all the membranes and allowed all of the radiolabeled C9 to enter the top of the running gel, it was assumed that the HMW C9 complexes (see Results) identified with 0.1% SDS represented the maximum incorporation of C9 into cell-bound membrane attack complexes detectable with an SDS-containing system. After electrophoresis the gels were soaked in 7% acetic acid/5% glycerol solution, dried and subjected to autoradiography at −80°C using Lightning-Plus Cronex intensifying screens (Dupont Co., Wilmington, DE) and Kodak XAR-2 film (Eastman Kodak Co., Rochester, NY). The autoradiograms were scanned using an ACD-18 Densitometer (Gelman Sciences, Inc., Ann Arbor, MI).

Results

Reactive Lysis. Table I summarizes data from six different experiments in which specific C9 binding to PNH E and normal E were compared, using the basic reactive lysis system described in Materials and Methods and conditions in which lysis is limited by C8 concentration. Shown in Table I are the two most commonly used inputs of C8. Similar comparative results were found with C8 inputs ranging from 6.5 to 99 ng/ml. Within a given experiment, lysis of PNH-III E was 2.5–4.2 times that of normal E for patient L and 4.5–5.8 times that of normal E for patient R. Nevertheless, the specific C9 binding per PNH E is equal to or less than that found for normal E. Therefore, the number of molecules of C9 bound per effective lytic site is considerably lower for PNH-III E than for normal E. The lysis of PNH-II E is only slightly greater than that of normal E and the C9 binding is slightly less, so that somewhat fewer C9 molecules are bound per lytic site by PNH-II E than by normal E.

Seven additional experiments using various modifications (see Materials and Methods) of the above "C5b-8 limited" reactive lysis protocol yielded varying absolute values for lysis and C9 binding, but the relationships between PNH and normal E remained similar to those shown in Table I. To facilitate comparisons between PNH and normal E throughout these 13 experiments, the data have been normalized by dividing values for lysis (Z), C9 binding (molecules per E), and C9 bound per lytic site (molecules per E per Z) obtained for PNH E by the corresponding values obtained for each normal E studied in the same experiment. The means (±SD) of the ratios representing each comparison are shown in Table II. PNH-III E from patient R lysed 5.27-fold more than normal E in the 24 experiments with <100% lysis, whereas PNH-III E from patient L lysed 3.1 times normal, and the average lysis of PNH-II E was less than twice normal. The C9 binding was equal to normal E for cells from patient R (type III) and from patient K (type II), but only 58% of that found on normal E for cells from patient L (type III). The last column in Table II presents the main finding, i.e., that only 22–25% as many bound C9 molecules were required to produce an effective

TABLE I
C8-dependent Binding of ^{125}I-C9 during Reactive Lysis of Normal Human and PNH Erythrocytes

Erythrocyte type	C8 input (ng/ml)*	Number of experiments	Lysis (Z)‡	Specific C9 binding§	
				Molecules per E	Molecules per effective lytic site
PNH-III (R)	52	5‖	3.05	333 ± 151¶	138
	13	5	2.45 ± 0.39¶	160 ± 86	35 ± 10¶
PNH-III (L)	52	5	2.39 ± 0.71	170 ± 47	77 ± 29
	13	5	1.68 ± 0.34	99 ± 31	58 ± 13
PNH-II (K)	52	5	1.38 ± 0.15	345 ± 68	250 ± 51
	13	6	0.71 ± 0.14	154 ± 70	215 ± 84
Normal (A)	52	3	0.77 ± 0.15	427 ± 66	559 ± 96
	13	4	0.45 ± 0.13	227 ± 69	516 ± 53
Normal (B)	52	5	0.77 ± 0.27	363 ± 88	535 ± 267
	13	6	0.42 ± 0.13	178 ± 71	485 ± 289
Normal (C)	52	5	0.96 ± 0.22	381 ± 147	429 ± 218
	13	6	0.52 ± 0.11	179 ± 87	368 ± 214
Normal (D)	52	1	0.62	147	236
	13	1	0.27	100	366

* Final concentration in reaction volume of 0.375 ml, containing 3×10^7 EC5b-7 and 91 ng/ml of ^{125}I-C9.

‡ $Z = -\ln(1 - \text{fraction of cells lysed})$. This corresponds to effective lytic sites per cell if a single complete C5b-9 site is assumed to be sufficient to lyse a cell.

§ Specific binding is determined by subtracting cpm bound in the absence of C8 from total cpm bound.

‖ PNH-III E (R) gave 100% lysis in 3 of 5 experiments using 52 ng/ml C8, and in 1 of 5 experiments using 13 ng/ml C8. Therefore, Z and C9 molecules per Z are calculated from only two values for the higher C8 concentration and from four values for the lower C8 concentration. C9 molecules per E reflects all five experiments.

¶ Values are the means ± SD (where appropriate).

hemolytic site on either sample of PNH-III E as on normal E. PNH-II E are less abnormal in this parameter.

Fig. 1 presents data from one of four C9 binding experiments performed with constant C5b-8 but a broad range of C9 inputs. All four experiments gave similar results. At each C9 input used, lysis was greater for PNH-III E than for normal E (Fig. 1 *A*), whereas specific C9 binding was similar for PNH and normal E (Fig. 1 *B*). Lysis of PNH E was higher than for normal E at all levels of C9 binding obtained (Fig. 1 *C*). Thus, lytic efficiency of bound C9 is higher on PNH-III than on normal E when C9 is the limiting reagent, as it is when C5b-8 sites are limiting (Tables I and II).

Next, we wished to determine whether specifically bound C9 was incorporated into HMW C5b-8$(C9)_n$ membrane attack complexes (MAC) with different efficiency on PNH E than on normal E. Fig. 2 shows an autoradiogram of an SDS-

TABLE II

Summary Comparison of PNH and Normal Erythrocytes in 13 Experiments: Ratios of Values for Reactive Lysis and Specific C9 Binding

Erythrocytes compared with normal	Lysis (Z)*	C8-dependent binding of ^{125}I-C9*	
		Molecules per E	Molecules per effective lytic site
Type III PNH (R)	5.27 ± 3.21 (24)‡	0.92 ± 0.23 (45)	0.25 ± 0.15 (24)‡
Type III PNH (L)	3.10 ± 1.38 (46)	0.58 ± 0.25 (46)	0.22 ± 0.11 (46)
Type II PNH (K)	1.76 ± 0.57 (41)	1.01 ± 0.30 (41)	0.60 ± 0.19 (41)

* See footnotes for Table I.

‡ Data are means ± SD of ratios calculated by dividing values obtained for PNH E by corresponding values for normal E in each of 13 experiments. The total numbers of comparisons in the 13 experiments are shown in parentheses. Data for lysis (Z) and C9 molecules bound per effective lytic site on type III PNH E (R) were compiled from fewer experiments than were available for calculating C9 binding because these cells lysed completely in some experiments, so that Z values could not be derived.

PAGE gradient slab (2.4–11% acrylamide) loaded with ghosts (solubilized in sample buffer containing 0.1% SDS) from one of the reactive lysis experiments summarized in Table I. At a given C8 input PNH and normal E membranes showed no obvious difference in the distribution of radiolabeled C9 among the three main regions of interest: the polydisperse HMW region, disulfide-linked dimer, and monomeric C9. The reduced overall C9 binding to PNH-III E of patient L (Tables I and II) is reflected in decreased radioactivity in all three regions. The HMW C9 complexes found on ghosts solubilized in 0.1% SDS also contain C7 (data not shown), and presumably represent MAC bearing various multiplicities of C9, accounting for their heterodisperse appearance in SDS-PAGE. No C7 was found in the region occupied by C9 dimers.

The results of densitometric quantitation of the distribution of ^{125}I-C9 radioactivity in the autoradiogram shown in Fig. 2 are presented in Table III. Lysis and total unfractionated C9 binding for each cell type in this experiment are shown for reference. As usual, total C9 binding on PNH E is equal to or less than that seen on normal E, whereas lysis is greater for PNH E. Also shown is the specific C9 binding in the HMW region of the autoradiogram, quantifying the incorporation of C9 into MAC. The absolute amount of HMW C9 on PNH E is similar to or less than that on normal E, and therefore, the number of HMW C9 molecules per effective hemolytic site is considerably smaller for PNH-III E than for normal E or PNH-II E, indicating increased hemolytic efficiency of MAC-associated C9 on PNH-III E. SDS-PAGE analysis of the experiments with limiting C9 gave similar results, although the HMW and dimer C9 bands were not visible on the autoradiographs of the tracks with the lowest C9 inputs, due to lack of sufficient counts on the gels.

Washed ghosts bearing ^{125}I-C9 were also dissolved in sample buffer containing 2% SDS (without reducing agent) before SDS-PAGE and autoradiography. Under these conditions most of the HMW ^{125}I-C9 complexes appeared to be dissociated into smaller complexes, dimeric ^{125}I-C9, and monomeric ^{125}I-C9. However, in multiple experiments some ^{125}I-C9 remained in a faint broad band in the highest molecular weight region, despite prior incubation in 2% SDS at

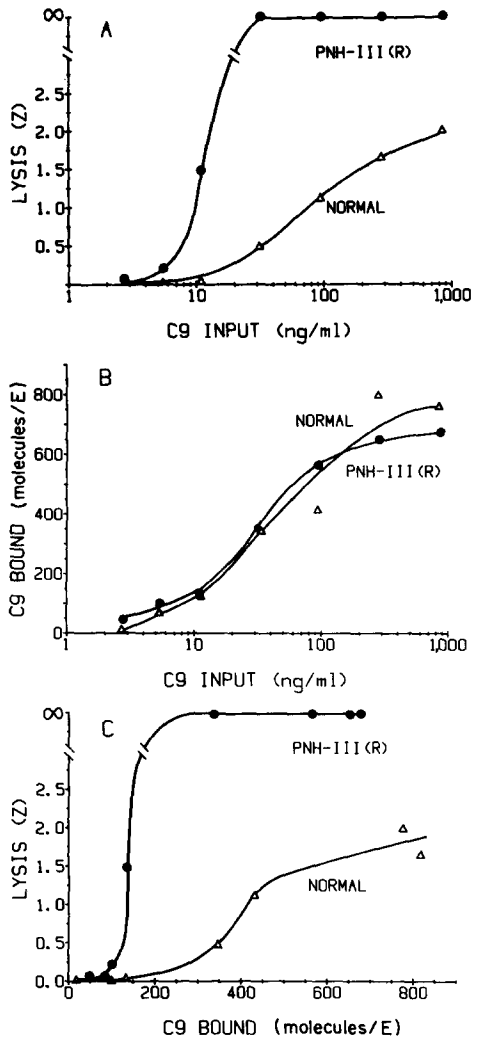

FIGURE 1. C9 binding during reactive lysis of PNH-III E (patient R) (●) and normal E (△) over a wide range of C9 inputs. EC5b-7 (1.2×10^7), prepared from each cell type with identical inputs of C5b6 and C7, were incubated (60 min, 37°C) with 1 μg/ml C8 and the indicated concentrations of ^{125}I-C9 in a final volume of 130 μl.

100°C for 1.5 min. These SDS-resistant very HMW C9 complexes were more apparent on PNH-III E (Fig. 3, lanes *1* and *2*) than on PNH-II E (lane *3*) or normal E (lane *4*) in 9 of 13 experiments, although the difference was often more subtle than that seen in Fig. 3. In four experiments, these complexes were similar on PNH and normal E but in no experiment were these SDS-resistant diffuse HMW C9 complexes shown in greater concentration on normal E than on PNH E. A few experiments used 0.5% SDS as well as 2%; the results were similar with both SDS concentrations.

In other experiments, the 2% SDS-resistant HMW C9 complexes appeared as sharp bands in the HMW region (mol wt ~800–1,000 × 10^3), without any

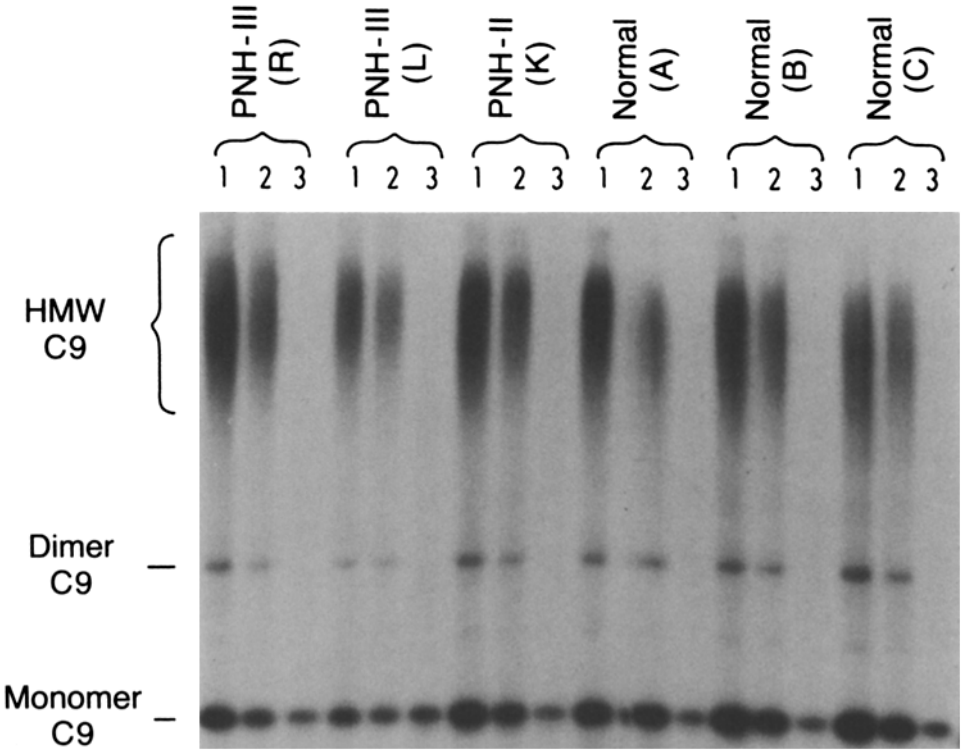

FIGURE 2. Autoradiogram showing the molecular weight distribution of ^{125}I-C9 on the membranes of PNH and normal E after reactive lysis. The washed membranes of complement-lysed and unlysed E were solubilized in sample buffer containing 0.1% SDS and analyzed by 2.4–11% acrylamide gradient slab SDS-PAGE. The C9 monomer, dimer, and HMW regions were verified by reference to radiolabeled mol. wt. markers (IgM, IgG, BSA; not shown). Each cell type is represented by three lanes corresponding to the C8 input: lane *1*, 52 ng/ml; lane *2*, 13 ng/ml; lane *3*, no C8.

difference between PNH and normal E (Fig. 3, lanes *5* and *6*). Most of the C9 dose-response experiments showed this sharp band, which did not appear to vary according to C9/C8 ratio. A few experiments with ^{125}I-C7 revealed a similar appearance of HMW C7 after dissolution of ghosts in 2% SDS (Fig. 3, lane *7*), indicating incorporation of C7 together with C9 into SDS-resistant HMW complexes during C lysis of human E, in agreement with Podack and Tschopp (7). In any case these sharp bands of HMW C9, when seen, did not distinguish between PNH and normal E in reactive lysis experiments.

Antibody-initiated Lysis by C1-C9. Having shown that C9 binding and incorporation into membrane-bound HMW complexes are, with the one exception just noted using 2% SDS, quantitatively similar on PNH and normal E during reactive lysis, we wished to study the same processes during classical pathway-mediated lysis, wherein the C3-amplifying effect of DAF deficiency could be expressed.

Table IV displays the lysis, C9 binding, and C9 incorporation into HMW complexes from a representative classical pathway experiment comparing PNH-III E and normal E. EAC1-7 were prepared from both cell types with antibody

TABLE III

Quantitation of C9 Incorporation into HMW Complexes on PNH and Normal Erythrocytes during Reactive Lysis

Erythrocyte type	C8 input (ng/ml)	Lysis (Z)	Total specific C9 binding		Specific C9 binding in HMW complexes*	
			Molecules per E	Molecules per effective lytic site	Molecules per E	Molecules per effective lytic site
Type III PNH (R)	52	∞ (100%)‡	381‡	—	284	—
	13	∞ (100%)	195	—	144	—
Type III PNH (L)	52	2.16	155	72	114	53
	13	1.78	94	53	66	37
Type II PNH (K)	52	1.59	373	234	215	135
	13	0.95	192	202	129	136
Normal (A)	52	0.94	435	463	209	222
	13	0.61	227	372	87	143
Normal (B)	52	0.81	414	509	231	284
	13	0.49	224	458	106	216
Normal (C)	52	0.99	460	467	240	244
	13	0.59	225	380	132	224

See footnotes for Tables I and II.

* Estimation by densitometric scan of autoradiograph shown in Fig. 2, in which membranes were solubilized in 0.1% SDS. The amount of radioactivity in the HMW region of each track in the autoradiogram is calculated by multiplying the fraction of total area under the relevant portion of the densitometry tracing by total cpm applied to the track. Specific binding is determined by subtracting HMW radioactivity of tracks representing EC5b-7 incubated with ^{125}I-C9 in the absence of C8.

‡ Values for lysis and total C9 binding are means of duplicate determinations.

and purified C1-C7, as described in Materials and Methods. In separate studies (not shown), antibody uptake by PNH and normal E was similar as measured by binding of radiolabeled human C1q. EC5b-7 were also prepared with C5b6 and C7 as in previously described experiments. When EAC1-7 and EC5b-7 intermediates were incubated with the same concentrations of C8 and radiolabeled C9, marked differences in C9 binding to PNH-III E were seen with the two intermediates. PNH EAC1-7 bound 13–20-fold more C9 than did PNH EC5b-7. This was accompanied by a marked increase in HMW C9 on PNH EAC1-7. The hemolytic efficiency of cell-bound C9 (and HMW C9) was markedly greater on PNH E during reactive lysis than during antibody-initiated classical pathway lysis in this and other experiments. In the experiment shown in Table IV, the minimal C8-dependent dose response for lysis of EAC1-7 suggests that another component, possibly antibody, was limiting. If so, some E (both PNH and normal) would not be susceptible to C9 binding and lysis, whereas superfluous C9 molecules could have been bound to the lysed EAC1-7. Although this would render the calculation of lytic efficiency for EAC1-7 less reliable, it does not alter

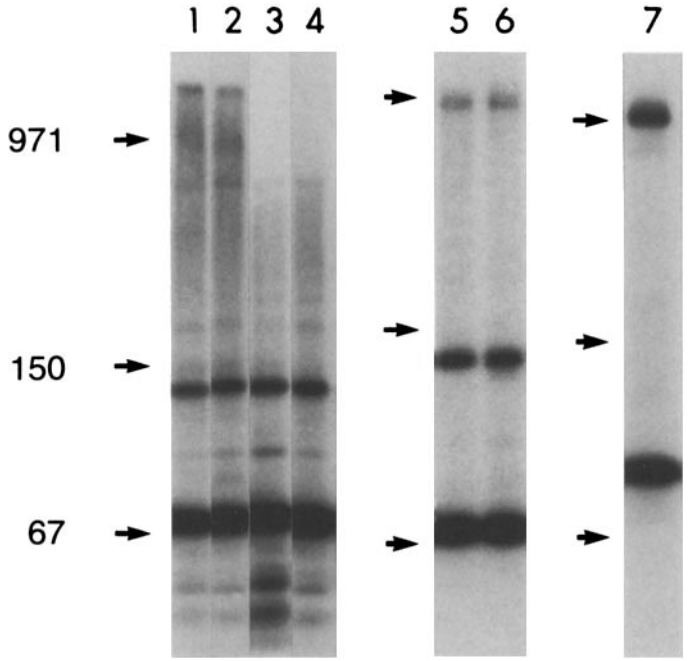

FIGURE 3. Composite of autoradiograms from three experiments in which E membranes were solubilized in sample buffer containing 2% SDS. Conditions for SDS-PAGE were otherwise as in Fig. 2. The mol. wt. markers ($\times 10^{-3}$) (*arrows*) designate the positions of radiolabeled IgM, IgG and BSA in each set of gels. Lanes *1–4* are from one of the ^{125}I-C9 binding experiments summarized in Table I, with 13 ng/ml C8 input. Lane *1*, PNH-III E (R); lane *2*, PNH-III E (L); lane *3*, PNH-II E (K); lane *4*, normal E. Lanes *5* and *6* compare the distribution of ^{125}I-C9 on PNH-III E (R) (lane *5*) and normal E (lane *6*) from another reactive lysis experiment. For comparison, the position of ^{125}I-C7 solubilized from PNH-III E membranes in a third experiment is shown in lane *7*.

the important conclusion of these experiments: that C9 binding and incorporation into HMW complexes is markedly greater on PNH-III E than on normal E during classical pathway lysis, in contrast to that seen with reactive lysis. These relationships are dramatically illustrated by the autoradiogram shown in Fig. 4. These findings are not surprising in view of the markedly increased C3b binding, and consequently enhanced C3/C5 convertase formation, known to occur on PNH E during classical pathway activation (1). In other experiments on classical pathway lysis using ^{125}I-C7 (not shown), excessive binding of C7 and incorporation into HMW complexes were also shown on PNH-III E as compared with normal E.

In experiments using a wide range of C9 concentrations in classical pathway-mediated lysis, C9 could be clearly shown to be limiting, thus allowing valid comparisons of hemolytic efficiency of bound C9 between PNH and normal EAC1-7. A representative experiment is presented in Fig. 5. As in reactive lysis, PNH-III E lyse to a greater extent than do normal E at each C9 input (Fig. 5*A*). In contrast to reactive lysis (Fig. 1), PNH-III E bind more C9 than do normal E at each C9 input, and this difference grows as C9 input increases (Fig. 5*B*). However, the lysis per C9 bound is similar for PNH and normal E over the

TABLE IV

Comparison of C9 Binding and Incorporation into High Molecular Weight Complexes on PNH and Normal Erythrocytes by Classical Pathway vs. Reactive Lysis

Cell intermediate tested*	C8 input (ng/ml)	Lysis (Z)	Total specific C9 binding		Specific C9 binding in HMW complexes‡	
			Molecules per E	Molecules per effective lytic site	Molecules per E	Molecules per effective lytic site
PNH EAC1-7	34	2.81	2,910	1,035	1,795	638
PNH EAC1-7	17	2.64	2,827	1,052	1,818	676
Normal EAC1-7	34	0.72	374	521	253	352
Normal EAC1-7	17	0.71	314	442	238	335
PNH EC5b-7	34	∞ (100%)	230	—	202	—
PNH EC5b-7	17	1.59	136	86	129	81
Normal EC5b-7	34	0.69	240	349	200	291
Normal EC5b-7	17	0.48	144	300	109	227

* EAC1-7 were generated by incubating type III PNH (R) or normal E with heat-inactivated human anti-Tj^a, purified guinea pig C1, human C4, and human C2-C7 (see Materials and Methods). EC5b-7 were generated with C5b6 and C7. Washed EAC1-7 or EC5b-7 (1.1×10^7/ml) were incubated (60 min, 37°C) with the indicated concentrations of C8 (for buffer alone for nonspecific binding) and ^{125}I-C9 (139 ng/ml).

‡ See footnotes for Table III. The ghosts applied to the gel used for this autoradiogram were solubilized in 0.1% SDS.

limited range of C9 binding achieved on normal E (Fig. 5*C*). Note the marked difference in the scale of C9 binding between Fig. 1 (reactive lysis) and Fig. 5 (classical pathway lysis).

Discussion

We have adapted a system for reactive lysis (18, 19), using purified human C5b6, C7, C8, and C9, that produces significant lysis of normal human E. With this system, we have previously shown that PNH-III E lyse 3–5-fold more than normal E at a given C input (5), and that this enhanced lysis is not accompanied by increased C7 binding (6). In this report, we extend the observations with this reactive lysis system to show that the enhanced lysis of PNH-III E is not accompanied by increased C9 binding (Tables I–III), or by overall increased incorporation of C9 into HMW membrane-bound complexes (Table III and Fig. 1), suggesting that cell-bound MAC-incorporated C9 is more efficient in producing lysis of PNH-III E than of normal E. There is a suggestion of a qualitative difference in the cell-bound MAC on PNH-III E, however. Our frequent (but not universal) finding of greater SDS-resistance of HMW C9 complexes on PNH-III E than on normal or PNH-II E (Fig. 3) may reflect the presence of a lytically relevant subset of C5b-9 complexes that are stabilized by interaction with membrane lipids. The apparent HMW of these complexes could reflect high C9 multiplicity, or they could represent SDS-resistant micelles containing membrane constituents as well as one or more MAC. The capacity of our current techniques

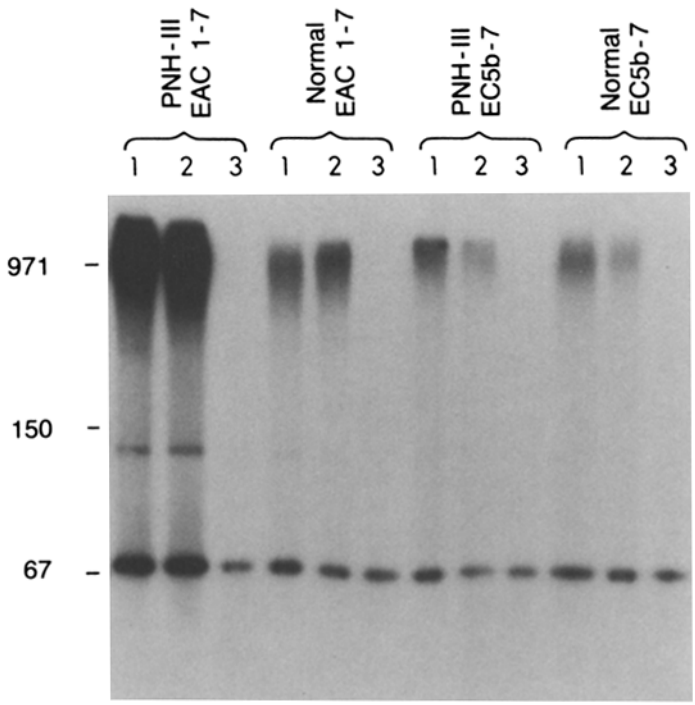

FIGURE 4. Autoradiogram of SDS-PAGE analysis of E membranes from the experiment shown in Table IV. Technical conditions were as described in the legend of Fig. 2, using 0.1% SDS to solubilize the membranes. Each cell intermediate is represented by three lanes, corresponding to C8 input; lane *1*, 34 ng/ml; lane *2*, 17 ng/ml; lane *3*, no C8. The positions of the mol. wt. ($\times 10^{-3}$) markers (radiolabeled IgM, IgG and BSA) are shown.

to resolve and identify these complexes may be borderline, but they are seen in a majority of our experiments. In preliminary work using membranes from complement-lysed PNH and normal E (separated from the unlysed E), these HMW SDS-resistant C9 complexes were seen on normal E as well as on PNH E. Our current techniques for separating lysed from unlysed E are insufficiently quantitative to allow us to determine whether these SDS-resistant C9 complexes are present in equivalent amounts on the lysed fractions of PNH and normal E in these experiments, but we are continuing work in this area. Nevertheless, these observations raise the possibility that the HMW C9 complexes resistant to dissociation by 2% SDS may represent the truly lytic configuration of bound C5b-9, and that this configuration may be favored by the PNH-III E membrane.

We have studied two PNH patients whose blood contains >95% type III E (in multiple blood samples from each patient), revealing interesting similarities and differences between these examples of type III E. E from patient R consistently lyse to a greater extent than those from patient L, and show greater C9 binding (Table I). E from patient L, while far more lytically sensitive than normal E or PNH-II E, consistently exhibit lower C9 binding than normal E. When compared with normal E, however, the lytic efficiency of C9 on both examples of PNH-III is very similar (Table II). These observations of consistent differences between two PNH-III E populations raise the possibility that two mechanisms may regulate

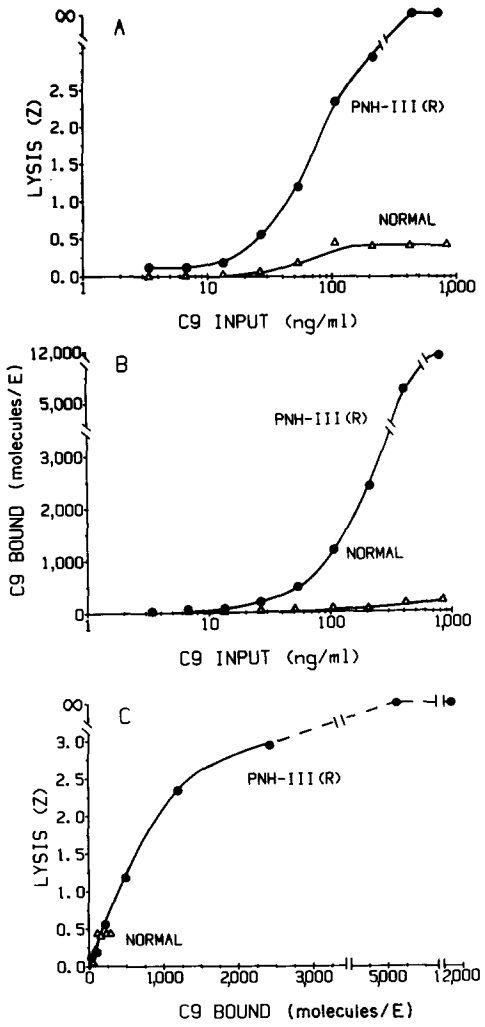

FIGURE 5. C9 binding during antibody-initiated lysis by C1-C9 of PNH-III E (●) and normal E (△) over a wide range of C9 inputs. EAC14 were prepared from each cell type with identical inputs of human anti-Tja, guinea pig C1 and human C4, and adjusted to 1.5×10^8/ml in $SGVB^{2+}$. 80 μl of cell suspension were mixed with 25 μl of a mixture containing purified human C2, 3, 5, 6, 7, and 8 plus 25 μl of dilutions of ^{125}I-C9 in $SGVB^{2+}$, producing the final C9 concentrations shown. After 60 min incubation at 37°, lysis and C9 binding were determined as in Materials and Methods. Nonspecific binding in the absence of C2-8 was subtracted from total binding.

the susceptibility of normal human E to lysis by human complement MAC: one modulating C9 binding to EC5b-8, and the other modulating the ability of C5b-9 to produce membrane lesions sufficient to lyse the cell. The latter mechanism might act to limit membrane insertion of MAC, to enhance closure of the channels formed by MAC, or to confer greater resistance to lysis by a given number of channels. PNH patients R and L may represent variations within the normal spectrum of C9 binding regulation, yet both are abnormal in their sensitivity to the lytic effect of bound MAC, suggesting that the latter abnormality

is dominant in its effect on overall susceptibility to complement lysis. Confirmation of these speculations will require further study.

In contrast, when antibody-sensitized E are lysed by classical pathway activation of purified C components, PNH-III E bind far more C9 (and C7) than do normal E. Much of this excess C9, however, is in a lytically less efficient form, so that the overall lytic efficiency of specifically bound C9 on PNH-III E is similar to (Fig. 5) or less than (Table IV) that on normal E.

We have also studied one patient (K) whose blood contains >90% PNH-II E. Because of overlap between type II and normal E in the complement lysis sensitivity test, this patient's blood could contain as many as 10% normal (type I) E without being detected by this test, but the lysis curves suggest that few or none of this patient's E are type III. Although we originally believed that this patient's type II E were normally sensitive to reactive lysis (5), our more extensive subsequent experiments have shown modestly increased sensitivity to reactive lysis of this patient's type II E. This is accompanied by normal C9 binding and modestly enhanced lytic efficiency of membrane-bound C9, suggesting that the PNH-II E of this donor may share the same defect as PNH-III E, but to a much lesser extent, or that our patient has some type III E not detected by the complement lysis sensitivity test.

Others have studied C7, C8, and C9 binding to PNH and normal E in different lytic systems, obtaining results that differ in some respects from ours. Parker et al. (20) studied lysis in C7-, C8-, or C9-depleted serum reconstituted with the appropriate radiolabeled complement protein, but they did not measure C9 (or other C component) incorporation into HMW complexes. When antibody-sensitized E (EA) were lysed in such reconstituted serum, the findings of Parker et al. resembled our own results with EA and purified C1-9 (Figs. 4 and 5), in that they saw increased C9 (and C8) binding to PNH-III EA at any given serum concentration. These workers found increased lysis of PNH EA for any observed quantity of C9 bound, whereas we found similar levels of lysis of PNH and normal EA per C9 bound during classical pathway-initiated lysis using purified C components with limiting C9 (Fig. 5 *C*). A more substantial difference between our results was seen when Parker et al. studied lysis of unsensitized E in EDTA-treated serum initiated by a fluid phase alternative pathway activator (cobra venom factor–Bb complex), which the authors equated with reactive lysis. In these experiments, PNH-III E bound large amounts of C7, C8, and C9, and lysed fully, whereas normal E bound undetectable amounts of C7, C8, and C9, and did not lyse; PNH-III E bound a somewhat greater number of C3 fragments than did normal E. Many methodological differences exist between the studies of Parker et al. and ours, but we cannot account for the striking differences between their results and our C5b6-initiated reactive lysis experiments by: (*a*) the method of radiolabeling C9; (*b*) the buffers used for the experiments; (*c*) the presence of C5b6, C7, C8, and C9 in the reaction mixture simultaneously; or (*d*) the method used to separate ghosts and E from unbound C9. We have attempted to reproduce all of the above conditions used by Parker et al. (20) with our purified components, and we still obtain similar C9 binding to PNH and normal E. Our ability to recover PNH E ghosts bearing very high amounts of radiolabeled C9 in the classical pathway experiments indicates that our standard method of

washing and recovering ghosts does not selectively lose PNH E ghosts bearing excess radiolabeled C9. Preliminary experiments suggest that the presence of reactive lysis inhibitors (lipoproteins and/or S protein) in the serum used in their experiments may, at least partially, account for the differences between our results and those of Parker et al. This is an area of ongoing investigation in our laboratory. It does seem quite clear that CVF-Bb-initiated lysis in whole serum is not equivalent to C5b6-initiated reactive lysis.

Hu and Nicholson-Weller (21), using a very different method of determining C9 binding, have recently found a single sample of PNH E to bind more C9 than normal E (but equal C7 and C8). Most of their experiments were done with antibody-sensitized E and whole serum, and these results are similar to ours using classical pathway lysis (except that they found equal C7 binding on PNH and normal E). In a single experiment using purified C5 through C9 activated by acid exposure, Hu and Nicholson-Weller found 5–6-fold more C9 bound to PNH than to normal E. They also reported finding more SDS-resistant polymerized C9 on PNH E, but equal efficiency of C9 insertion on PNH and normal E; however, their report does not state whether this experiment was done with classical antibody-serum treatment or with acid-activated purified C5-C9. If the classical pathway of complement was used for the latter two experiments, their results are consistent with ours, since we find increased C9 binding and C9 polymerization, but normal or decreased lytic efficiency of bound C9 on PNH-III E by this mechanism. We cannot fully account for the other differences between their results and ours, but the experimental systems used are very different.

Although it seems clear that we and others do find markedly increased C9 binding to PNH-III E by some lytic mechanisms, it is equally clear from our very reproducible findings with reactive lysis initiated by purified C5b6 that PNH-III E can show markedly greater lysis than normal E with equal (or even less) specific C9 binding. Thus, there presumably exist some control mechanism(s) on normal human E that can restrict lysis without restricting the amounts of C7 or C9 bound, and these mechanism(s) appear to be deficient on PNH-III E. It is possible that other mechanisms, perhaps interacting with control proteins of serum, may limit C7 and C9 binding to human E, but these alone cannot account for the enhanced sensitivity to reactive lysis of PNH-III E.

Summary

As part of a broader analysis of the mechanism(s) by which the most sensitive (type III) paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes are excessively sensitive to reactive lysis by isolated C5b6, C7, C8, and C9, we have compared type III PNH (PNH-III) and normal human E in respect to both total specific binding of ^{125}I-C9 and the proportion of cell-bound C9 appearing in high molecular weight (HMW) complexes. In a previous report, we found that after exposure to purified C5b6 and ^{125}I-C7, specific C7 binding and, by implication, EC5b-7 formation were equal for PNH-III E and normal E. In the present study, C8-dependent binding of ^{125}I-C9 to PNH-III EC5b-7 and normal EC5b-7 was also similar, although lysis of the PNH-III E was up to five times greater; that is, PNH-III E required fewer bound C9 molecules to produce an effective

lytic site than did normal E. To quantify radioactivity in monomeric and HMW forms of membrane-bound C9, lysed and unlysed E were subjected to low ionic strength buffers to convert all E to ghosts. These ghosts were solubilized in 0.1 or 2% SDS (without reduction) and electrophoresed on 2.4–11% polyacrylamide gradient gels followed by autoradiography and densitometric scanning. With 0.1% SDS, broad, heterodisperse zones of HMW C9 were recovered from both PNH and normal ghosts; the amounts of C9 incorporated into the HMW complexes were similar for PNH-III E and normal E. In selected experiments, ^{125}I-C7 could be shown in these same HMW bands. When membranes were solubilized in 2% SDS, the overall proportion of HMW C9 complexes compared with dimer and monomer C9 was reduced on both PNH and normal membranes. In many, but not all experiments, more of the highest mol wt C9 complexes were detected from PNH-III E membranes solubilized in 2% SDS than from normal or PNH-II E membranes similarly treated.

When antibody-sensitized E were lysed by purified C1-C9, PNH-III EA bound far more C9 than did normal EA, and both lysis and C9 incorporation into HMW complexes were markedly and proportionately increased over normal; however, lytic efficiency of ^{125}I-C9 bound to PNH EA was equal to or less than that bound to normal EA.

These observations indicate that the exaggerated sensitivity of PNH-III E to the isolated membrane attack sequence (C5b-9) does not require supranormal C9 binding; nor was preferential formation of HMW forms of C9 (i.e., as poly-C9 or C5b-9 complexes) detected on PNH E when 0.1% SDS was used to solubilize the membranes. The lytic differences between these cell types could still involve differing efficiency in formation on the PNH E membrane of a subset of especially lytically effective C5b-9 complexes, or might involve differences in membrane-damaging events subsequent to C5b-9 assembly. The putative highly lytic subset of C5b-9 complexes, or the subset of membrane-bound C5b-9 complexes actually inserted in the lipid bilayer, might be those detected in some experiments as the HMW complexes with greater resistance to 2% SDS.

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