Complement Fixation by Model Immune Complexes Free in Solution and Bound onto Cell Surfaces†

David M. Segal,* Ruth L. Guyer, and Paul H. Plotz

ABSTRACT: Immunoglobulin (IgG) molecules with anti-2,4-dinitrophenyl activity were covalently cross-linked by using three cross-linking reagents. The resulting oligomers were separated into monomer, dimer, trimer, and heavy fractions. These stable assemblages of IgG molecules were incapable of fixing dilute whole guinea pig complement in solution. When oligomers were further aggregated noncovalently into larger complexes, all were able to fix complement. Radioiodinated oligomers were attached to 2,4,6-trinitrophenylsulfonic acid treated sheep red blood cells (N_3ph-SRBC), and the number of bound molecules was determined from the cell-associated radioactivity. Complement-mediated lysis of N_3ph-SRBC was then assayed over a range of levels of bound protein and at increasing concentrations of complement. The lytic efficiencies of all oligomers increased with the number of bound molecules, with complement concentration, with hapten density on N_3ph-SRBC, and with oligomer size. The results suggest that two adjacent IgG molecules may not serve as a unit signal for triggering the complement cascade, but, instead, initiation occurs with increasing efficiency as the size of cell-bound IgG clusters increases.

Interactions between antibodies of the IgG[1] class and specific antigens in the presence of serum can lead to complement fixation. Under special circumstances, monomeric IgG has been reported to fix complement [e.g., Goers et al. (1974), Augener et al. (1971), and Pecht et al. (1977)]. However, most of the available data are consistent with the view that the normal mechanism of complement fixation involves formation of multivalent aggregates of IgG (Metzger, 1978). Conflicting values have been obtained for the minimum size of a complex necessary and sufficient for promoting the binding and activation of serum complement. In some studies, two IgG molecules in close proximity were sufficient for fixation (Cohen, 1968; Borsos & Rapp, 1965; Ishizaka et al., 1965), while in other systems larger aggregates were needed (Hyslop et al., 1970; Jaton et al., 1976). In all of these studies, the immune complex models lacked homogeneity and/or stability with respect to size.

In order to circumvent these problems, we have produced stable, covalently cross-linked oligomers of IgG antibodies using bivalent affinity labeling reagents (Segal & Hurwitz, 1976; Plotz, 1977; Plotz et al., 1979). The resulting oligomers are cross-linked through their antigen combining sites, and they are well defined with respect to size. Oligomers of similar sizes have also been produced by the nonspecific cross-linking of IgG antibodies with a bivalent active ester (Segal & Titus, 1978). In this paper, data are presented describing the complement fixing and hemolytic activities of these oligomers.

Materials and Methods

Sources of Reagents. AG1-X8 (200–400 mesh) anion-exchange resin was obtained from Bio-Rad (Richmond, CA), Ultrogel AcA 22 and AcA 34 were from LKB (Rockville, MD), Sephacryl S-200 was from Pharmacia (Piscataway, NJ), and diethylaminoethylcellulose DE52 was from Whatman, Inc. (Clifton, NJ). 1-Fluoro-2,4-dinitrobenzene was obtained from the Eastman Organic Chemical Co. (Rochester, NY), dimethylsuberimidate was from the Pierce Chemical Co. (Rockford, IL), and picrylsulfonic acid was from the Aldrich Chemical Co. (Milwaukee, WI). ^{125}I was purchased from NEN (Boston, MA) as the "low-pH solution". Bovine serum albumin (BSA) was purchased from Miles (Pentex, Kankakee, IL). Sheep red blood cells (SRBC) were obtained from the National Institutes of Health animal farm as whole sheep blood mixed 1:1 with Alsever's solution. Rabbit hemolysin and guinea pig complement were purchased from GIBCO (Grand Island, NY).

Preparation of Antibody Molecules. Rabbit anti-N_2ph antibodies were raised by immunizing New Zealand White rabbits with N_2ph-Keyhole limpet hemocyanin in complete Freund's adjuvant as previously described (Segal & Hurwitz, 1976). For IgM antibodies, rabbits were bled 5 days after the initial injection. For IgG antibodies, a booster injection was given 3 weeks after the first injection, and antibodies were isolated from serum collected at weekly intervals thereafter. For both types of antibodies, sera were made 0.01 M in EDTA (by addition of one-tenth volume of 0.1 M EDTA) prior to purification. This prevented monomeric immunoglobulin from being anticomplementary in the absence of added antigen (Hurwitz, Guyer and Segal, unpublished experiments).

IgG antibodies were isolated by affinity chromatography on a N_3ph-Sepharose 4B column as described previously (Segal & Hurwitz, 1976). The column was equilibrated with 0.01 M sodium acetate, 0.01 M EDTA, and 0.15 M NaCl, pH 5.5–6.0; after application of antiserum, the column was washed extensively with this buffer. Specific antibodies were eluted with 0.01 M N_2ph-glycine (pH 6.5–7.0) in a volume equal to the original sample volume. Hapten was removed with AG1-X8 (200–400 mesh) anion-exchange resin. Protein was concentrated by vacuum dialysis and applied to a column of Ultrogel AcA 22 or Sephacryl S-200 (2.6 × 90 cm) in 0.02 M borate-buffered saline, pH 8.5. The monomeric IgG peak was pooled and concentrated.

IgM antibodies would not elute from the affinity column and were thus isolated as the total IgM fraction. The antiserum (30 mL with 3 mL of 0.1 M EDTA) was made 40% saturated with $(NH_4)_2SO_4$, and the resultant precipitate was

† From the Immunology Branch, National Cancer Institute (D.M.S.), and the Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases (R.L.G. and P.H.P.), National Institutes of Health, Bethesda, Maryland 20014. *Received May 3, 1978; revised manuscript received December 29, 1978.*

[1] Abbreviations used: BDPE, bis(2,4-dinitrophenyl)pimelic ester; BSA, bovine serum albumin; di-BADL, bis(α-bromacetyl-ϵ-N_2ph-Lys-Pro)-ethylenediamine; DMS, dimethylsuberimidate; N_2ph, 2,4-dinitrophenyl; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G; IgM, immunoglobulin M; PBS, phosphate-buffered saline; SRBC, sheep red blood cells; N_3ph, 2,4,6-trinitrophenyl; VBSG, veronal-buffered saline plus gelatin.

dissolved in 0.055 M Tris–phosphate, pH 6.0. The protein was dialyzed against three changes of this buffer and applied to a 2.5 × 30 cm DE52 column equilibrated with the 0.055 M Tris–phosphate buffer. Protein was eluted with the starting buffer; when the absorbance at 280 nM fell below 0.1, a linear gradient to 0.5 M Tris–phosphate, pH 5.1, was begun. The IgM eluted in a broad peak shortly after the gradient was started (at 2.8–5.4 mmho) and was identified by its ability to agglutinate N_3ph-coated sheep erythrocytes. The IgM was concentrated by vacuum dialysis and applied to a 2.6 × 90 cm Ultrogel AcA22 column in 0.02 M borate-buffered saline, pH 8.5. The IgM fraction eluted behind the exclusion volume and well in front of the major peak which eluted in the position of IgG. Protein in the IgM peak was pooled and concentrated. On polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the protein migrated as several closely spaced bands at approximately the position of a standard, purified monoclonal IgM protein from a patient with Waldenstrom's macroglobulinemia. Following reduction and alkylation, the IgM bands migrated at approximately the same rate as IgM monomeric subunits, light chains, and heavy chains.

Anti-sheep red cell antibodies were isolated from commercial hemolysin. The rabbit antiserum was precipitated with ammonium sulfate as were the anti-N_2ph antibodies. The precipitate was redissolved in and dialyzed against 0.0175 M Tris–phosphate, pH 6.8, and was applied to a 2.5 × 30 cm DE52 column equilibrated with this buffer. The "breakthrough" peak was concentrated and fractionated on an Ultrogel AcA34 column, yielding purified IgG antibody. The DE52 column was then equilibrated with 0.055 M Tris–phosphate, pH 6.0, and the IgM was fractionated as described above.

Preparation of N_2ph-BSA. N_2ph-BSA was prepared by incubating a 5% solution of BSA in 0.1 M Na_2CO_3 with a large molar excess of 1-fluoro-2,4-dinitrobenzene for 24 h at room temperature. After extensive dialysis, the degree of substitution was determined from the absorbance at 365 nM [assuming $E_m^{365} = 1.8 \times 10^4$ for the N_2ph group (Ashman & Metzger, 1969)] and from the protein concentration determined by the method of Lowry et al. (1951).

Affinity Cross-Linked Oligomers. The antibodies were specifically cross-linked through their antigen binding sites by using two bivalent affinity labeling reagents. (a) Anti-N_2ph antibodies were cross-linked with bis(N_2ph)pimelic ester (BDPE) at a 2:1 molar excess of BDPE/protein and at a protein concentration of 3 mg/mL. We refer to these as the "BDPE oligomers". (b) Anti-N_2ph antibodies were also cross-linked with bis(α-bromacetyl-ϵ-N_2ph-Lys-Pro)ethylenediamine (Segal & Hurwitz, 1976), yielding "di-BADL oligomers".

Nonspecifically Cross-Linked Oligomers. Monomeric anti-N_2ph antibodies were cross-linked nonspecifically through the ϵ-amino groups of lysyl residues on adjacent molecules by using dimethylsuberimidate (Segal & Titus, 1978). The oligomers formed with this reagent are referred to as "DMS oligomers".

Isolation of Oligomers. The oligomers of anti-N_2ph IgG prepared in these three ways were sorted into monomer, dimer, trimer, and heavy fractions on tandem Sephadex G-200 and Ultrogel AcA 22 columns (Segal & Hurwitz, 1976) in 0.02 M borate-buffered saline, pH 8.5, for the BDPE and DMS oligomers and in borate-buffered saline containing 1 mM ϵ-(N_2ph-amino)caproic acid for the Di-BADL oligomers.

Iodination. Oligomers were radiolabeled with ^{125}I by using the chloramine-T method as described previously (Segal & Hurwitz, 1977). The molar ratio of chloramine T/protein was (1.5–2.0):1. Proteins were labeled with approximately 1 mol of ^{125}I/mol of IgG. For IgM, the molar ratio of chloramine-T/^{125}I used was 4:1 (with the resulting ratio of ^{125}I/protein being 6:1).

Complement Fixation Assays. Direct complement fixation by oligomers was characterized by using a standard hemolytic assay (Wasserman & Levine, 1961). Oligomer, in 0.2 mL of veronal-buffered saline with 0.1% gelatin [VBSG, as described by Mayer (1961)], was mixed with 0.7 mL of VBSG and 0.2 mL of a 1:160 dilution of guinea pig complement (1.6 CH_{50} units) at 0 °C. The mixture was allowed to incubate overnight at 4 °C, and 0.2 mL (7×10^7 cells/mL) of hemolysin-coated SRBC (Mayer, 1961) was added. After incubation at 37 °C for 1 h, the cells were centrifuged, and the fraction of cells lysed was determined by measuring hemoglobulin release. In some experiments, 0.2 mL of antigen [$(N_2ph)_{16}$BSA] or hapten (ϵ-(N_2ph-amino)caproic acid) was preincubated with antibody for 30 min before addition of complement. The total volume of the reaction mixture was kept the same as that for assays without antigen by reducing the amount of VBSG to 0.5 mL.

Lysis of N_3ph-SRBC by Oligomers and Complement. (1) Preparation of N_3ph-Coated SRBC. SRBC were centrifuged at 1000*g* for 10 min and washed twice with 0.0067 M phosphate-buffered saline, pH 7.2 (PBS), and the packed cells were diluted 20-fold (5% cells) in PBS. Fifty milliliters of 5% SRBC and 50 mL of 2,4,6-trinitrobenzenesulfonic acid (at the desired concentration) in PBS were added to 400 mL of PBS, and the mixture was incubated 30 min at 37 °C. The cell suspension was centrifuged at 1000*g* for 10 min, washed once in VBSG, and resuspended to 5% N_3ph-SRBC in VBSG.

(2) Binding of Oligomers to N_3ph-SRBC. N_3ph-SRBC (0.15 mL of 5% cells) were mixed with 0.15 mL of dilutions of ^{125}I-labeled oligomer in VBSG. After 1-h incubation at 37 °C, cells were washed twice with 5 mL of VBSG and were resuspended in 1.2 mL of VBSG. Triplicate 100-μL aliquots were sedimented through oil to separate cell-bound antibody from free antibody (Segal & Hurwitz, 1977). The number of molecules of antibody bound per cell (N) was calculated from the ratio (R) of cell-associated radioactivity to the optical density at 413 nM of a 0.1-mL aliquot of cells added to 1.2 mL of H_2O. The formula used for the calculation was

$$N = \frac{(6.02 \times 10^{20})(4.88 \times 10^{-8})}{(\text{sp act.})} R$$

where sp act. is the specific activity of the oligomer in cpm/mmol and 4.88×10^{-8} is the OD/cell when lysed in H_2O as described.

(3) Complement-Mediated Lysis of N_3ph-SRBC with Attached Anti-N_2ph Oligomers. Samples of N_3ph-SRBC containing increasing amounts of bound oligomer and one containing no oligomer were incubated with adsorbed complement in the following manner. Complement (0.2 mL) was added to 1.0-mL aliquots of VBSG containing 0.1 mL of oligomer-coated SRBC. Cells and complement were incubated for 45 min at 37 °C and centrifuged, and the percent lysis was determined from the absorbance of the supernatant at 413 nm (hemoglobin release). Values for 100% lysis were determined from samples in which 0.1 mL of cells was added to 1.2 mL of H_2O, and 0% lysis was determined from samples in which 0.1 mL of cells was added to 1.2 mL of VBSG. The absorbance of the latter never deviated significantly from the absorbance of VBSG alone. Corrections were applied for the absorbance of complement. When several oligomers were being compared, all experiments were performed on the same

Table I: Inability of Oligomers of Rabbit IgG Anti-N_2ph Antibodies to Fix Guinea Pig Complement in Solution[a]

type of cross-linking	oligomer size	highest no. of μg giving 0–10% lysis	highest no. of μg tested	% lysis at highest no. of μg tested
DMS	1	111	111	0
	2–3	105	105	0
	4–6	39	117	15
	6–8	19	58	15
di-BADL[b]	1	200	200	0
	2	200	200	0
	3	200	200	5
	3–5	61	182	47
BDPE	1	204	204	0
	2	68	204	29
	3	128	128	0
	3–5	80	80	0

[a] Oligomers were incubated overnight at 4 °C with 1.6 CH_{50} units of whole guinea pig complement and then tested for fixation, as described in the text. [b] di-BADL oligomers were tested in the present of ε-(N_2ph-amino)caproic acid. Stock oligomer solutions (1 mg/mL) were made 10^{-4} M in hapten. Hapten and antibody were diluted and tested for fixation. Corrections were made for absorption of hapten at 413 nM.

batch of N_3ph-SRBC and with aliquots from the same absorbed complement sample.

(4) Absorption of Guinea Pig Complement. Twenty milliliters of frozen or lyophilized and reconstituted guinea pig serum was passed through a N_2ph-lysyl-Sepharose column (1.6 × 20 cm) at 4 °C equilibrated with VBSG. Tubes containing complement activity were pooled and diluted to 40 mL with VBSG, and aliquots were frozen at −70 °C.

Results

Description of Oligomers. The three cross-linking reagents produced oligomers with somewhat different antigen binding properties. These differences dictated the types of experiments which could be carried out with each. The BDPE oligomers are cross-linked through their antibody sites, and N_2ph-OH is liberated when the covalent bond is formed between reagent and antibody. After the oligomers are separated from the weakly binding hapten by gel filtration, the antibody sites are available for binding to external antigens. The di-BADL oligomers are also polymerized through site-specific linkages. Their antigen-binding sites remain mostly occupied, so that the oligomers have lost the ability to bind external antigens. Because the di-BADL oligomers aggregate into larger complexes when free hapten is not present in the solution (Segal & Hurwitz, 1976), these oligomers were handled in solutions containing 10^{-4} M (ε-N_2ph-amino)caproic acid. The DMS oligomers are cross-linked through neighboring lysines not necessarily in the antigen-binding regions (Hunter & Ludwig, 1972) and retain N_2ph and N_3ph binding capabilities when prepared from anti-N_2ph antibodies.

Complement Fixation by Oligomers in Solution. Monomer, dimer, trimer, and heavy fractions of the three types of oligomer preparations were tested for their ability to fix complement. Under the conditions used in these experiments, 1.6 CH_{50} units is completely fixed by 2–10 μg of standard, control antiN_2ph antibodies complexed with specific antigen (N_2ph-BSA); without antigen 200 μg of anti-N_2ph antibodies fixes very little or no complement. The oligomer fractions behaved like the uncomplexed, monomeric antibody. The data in Table I show that, for most of the oligomers, high levels of protein give only minimal (0–10%) levels of complement

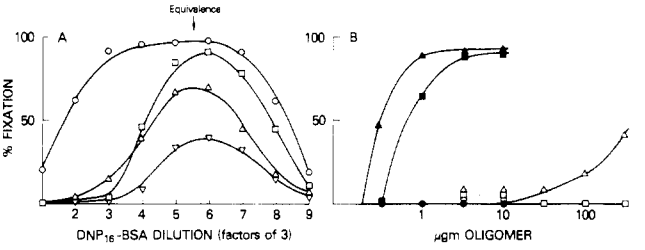

FIGURE 1: Fixation of whole guinea pig complement by oligomers of IgG anti-N_2ph antibodies. (A) BDPE oligomer (15 μg) with varying amounts of $(N_2ph)_{16}$BSA. The dilution of antigen at which the N_2ph groups are equal to the number of combining sites is marked "equivalence". (○) Monomer; (□) dimer; (▽) trimer; (△) heavy fraction. (B) Dilutions of di-BADL oligomers. Open symbols: oligomer solutions which contained 10^{-4} M ε-(N_2ph-amino)caproic acid before dilution. Closed symbols: oligomer dilutions were made in the absence of hapten. (○) Monomer; (□) dimer; (△) trimer.

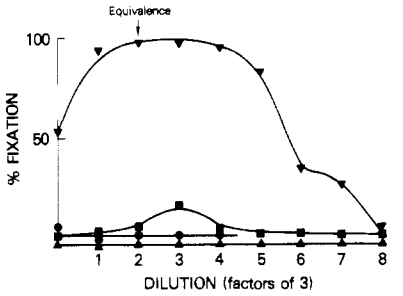

FIGURE 2: Complement fixation by BDPE dimer. (●) Fixation as a function of dimer concentration in the absence of hapten or antigen. Threefold dilutions of the dimer were tested, beginning with 200 μg/sample. (▼) Dimer (15 μg) was incubated with dilutions of $(N_2ph)_{16}$BSA and tested for fixation. (▲) Dimer (15 μg) was incubated with dilutions of ε-(N_2ph-amino)caproic acid (N_2ph concentration same as that of N_2ph on $(N_2ph)_{16}$BSA) and tested for fixation. (●) Dimer (15 μg) was incubated in the presence of $(N_2ph)_{16}$BSA and a threefold molar excess of N_2ph on ε-(N_2ph-amino)caproic acid.

fixation (third column). In the few cases in which fixation equal to or above 15% could be measured, the number of micrograms of protein required was large (columns four and five). For example, 47% fixation was obtained by using 182 μg of the di-BADL heavy fraction and 29% was measured for 204 μg of the BDPE dimer.

DMS oligomers and BDPE oligomers were subsequently tested for complement fixation in solutions containing a large multideterminant antigen [$(N_2ph)_{16}$BSA] with which the available antibody sites could react. The oligomer fractions were preincubated with $(N_2ph)_{16}$BSA before addition of complement, and the percent fixation was determined as described under Materials and Methods. Figure 1A shows the fixation curves for BDPE oligomers; similar results were obtained with the DMS oligomers (data not shown). The shapes of these curves, like precipitin curves, reflect the effect on complement fixation of changes in the stoichiometry of antigens and antibodies in the complexes (Osler et al., 1948). Oligomers formed with the di-BADL reagent do not retain antigen-binding capability and could not be assembled into larger aggregates by antigen. However, when hapten is removed from the di-BADL oligomers, aggregations occurs (Segal & Hurwitz, 1976), and complement fixation can then be detected in the 1–10-μg range (Figure 1B, closed symbols).

Figure 2 shows the effect of a small hapten, ε-(N_2ph-amino)caproic acid, on complement fixation by BDPE dimers. When hapten alone is added, the dimer does not fix complement. This means that the combination of a filled antigen-binding site and dimerization is not sufficient to elicit

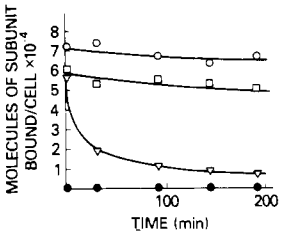

FIGURE 3: Binding of ^{125}I-labeled oligomers to N_3ph-SRBC as a function of time. Cells (modified with 0.24 mM N_3ph-sulfonic acid) were incubated 1 h at 37 °C with oligomers, centrifuged, and washed once with VBSG. Cells were then resuspended in VBSG at 37 °C, and timing was begun. (○) DMS dimer–trimer mixture; (□) DMS monomer; (▽) DMS dimer–trimer mixture with 10^{-4} M ε-(N_2ph-amino)caproic acid (added at 0 time); (●) DMS monomer incubated with N_3ph-SRBC in the presence of 10^{-4} M ε-(N_2ph-amino)caproic acid.

complement fixation. When both $(N_2ph)_{16}$BSA and the hapten are added, the dimer again does not fix complement, presumably because the hapten competes with the multivalent antigen for the antigen-binding site and prevents antigen-induced polymerization. Only when dimer is mixed with antigen in the absence of hapten does significant fixation occur.

Binding of Oligomers to N_3ph-Substituted Sheep Red Blood Cells. DMS and BDPE oligomers could bind to N_3ph-SRBC through their free antigen-binding sites. The attachment of radioiodinated oligomers to N_3ph-SRBC was measured as described under Materials and Methods and was observed to increase as the concentration of added oligomer increased. Attachment was not strictly proportional to the amount of added free oligomer and varied with the density of N_3ph groups on the SRBC. When the logarithms of the numbers of antibody molecules bound per cell were plotted against the logarithms of the dilutions of added antibody, straight lines were obtained. The slopes of these lines increased from 0.4 to 0.7 with increasing amounts of N_3ph for monomeric IgG anti-N_2ph antibodies, and the slope was 0.88 for anti-SRBC IgG antibodies at all N_3ph densities. When one oligomer fraction (DMS tetramer–hexamer) was tested for binding at three different specific activities of oligomer (prepared by adding unlabeled oligomer to ^{125}I-labeled oligomer), no differences could be observed in the binding curves. Thus, iodination did not interfere with the interaction of oligomers with N_3ph-SRBC.

The oligomers remained stably associated with the N_3ph-SRBC (Figure 3). After sensitizing the cells with oligomers, the unbound protein was washed away from the haptenated cells. During subsequent incubation at 37 °C, less than 17% of the cell-bound monomer and 7% of the dimer–trimer fraction of DMS oligomers were released from the cells over a period of 200 min. The attachment of oligomers to N_3ph-SRBC involved a specific antigen–antibody interaction, since hapten [ε-(N_2ph-amino)caproic acid] could totally inhibit binding of monomer to cells. Similarly, hapten could chase the dimer–trimer fraction from cells to which these oligomers were previously bound.

The data of Figure 3 are plotted in terms of the total number of 7S IgG "subunits" bound per cell rather than molecules of oligomer. By evaluating the data in terms of subunits, we can make direct comparisons of the binding efficiencies of oligomers of different sizes.

Complement-Mediated Lysis of Oligomer-Coated SRBC. Lysis of N_3ph-SRBC with attached oligomers was effected by addition of complement. In preliminary experiments (Figure 4A), the extent of cell lysis was shown to be unaffected by the specific activity of the oligomer preparation. Thus, lysis,

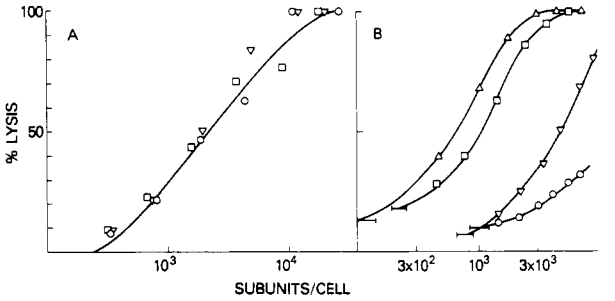

FIGURE 4: Complement-mediated lysis of oligomer-coated N_3ph-SRBC. (A) Lysis of N_3ph-SRBC (modified with 0.24 mM N_3ph-sulfonic acid) coated with a mixture of DMS tetramers, pentamers, and hexamers. Cells were coated with ^{125}I-labeled oligomers at three specific activities and treated with an eightfold dilution of guinea pig complement. Radiolabeled oligomer was added (○) undiluted, (□) diluted to 1/3 specific activity, and (▽) diluted to 1/9 specific activity with unlabeled oligomer. (B) Lysis of N_3ph-SRBC with BDPE oligomers and 40 CH_{50} units of complement. (○) monomer; (▽) dimer; (□) trimer; (△) heavy fraction (tetramer–hexamer). (—) Indicates lysis by complement in the absence of added antibody.

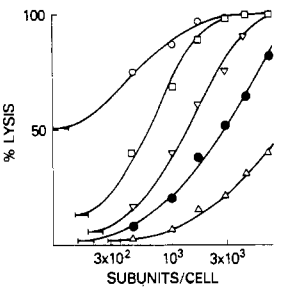

FIGURE 5: Lysis of N_3ph-SRBC coated with BDPE heavy fraction as a function of complement concentration. N_3ph-SRBC were modified with 0.24 mM N_3ph-sulfonic acid. (○) Complement undiluted (80 CH_{50} units titrated with hemolysin-coated SRBC); (□) complement diluted 1:2; (▽) complement diluted 1:4; (●) complement diluted 1:8; (△) complement diluted 1:16. (—) Indicates lysis by complement in the absence of added antibody.

like attachment, was unaltered by the iodination procedure. In another preliminary experiment (data not shown), it was observed that untreated monomeric anti-N_2ph IgG antibodies and the monomeric protein isolated from a BDPE oligomer preparation were equally efficient at mediating lysis of N_3ph-SRBC. This suggested that the conditions used in both the cross-linking and isolation procedures also did not alter the lytic properties of rabbit IgG antibodies.

Complement-mediated lysis of N_3ph-SRBC coated with BDPE oligomers is shown in Figure 4B. Lysis increased with the number of molecules of subunit bound per cell and with the size of the oligomer tested. Thus, for a given number of bound subunits, the heavy fraction was the most effective lytic agent, and the percent of lysis decreased with decreasing oligomer size. Because subunits represent the total number of constituent IgG molecules being tested, these data indicate that IgG molecules are most effective in initiating lysis when they are bound as large aggregates, and their efficiency is less for smaller clusters and least for singlets.

For oligomers of all sizes, lysis increased as the complement concentration increased. Representative data are shown in Figure 5 for lysis of N_3ph-SRBC coated with BDPE "heavy" oligomers at five complement concentrations. The number of IgG subunits required to attain a given degree of lysis decreased with increasing complement concentration. However, at high complement concentrations, lysis occurred in the absence of added antibody, an effect recently studied by Loos & Thesen (1978). Because of this artifact, the maximum amount of complement which could be used in these

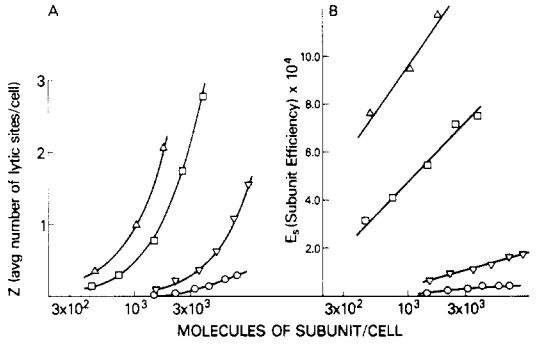

FIGURE 6: Lytic units formed by BDPE oligomers. (A) Data of Figure 4B corrected for multiple lytic sites. Z is calculated for a "one-hit" model (see text). Data of Figure 5A plotted in terms of E_s, the average number of lytic sites per cell divided by the average number of subunits bound per cell (Z/N_s). This parameter compares the efficiencies of IgG subunits in initiating lysis when bound as the (○) monomer, (▽) dimer, (□) trimer, or (△) heavy fraction.

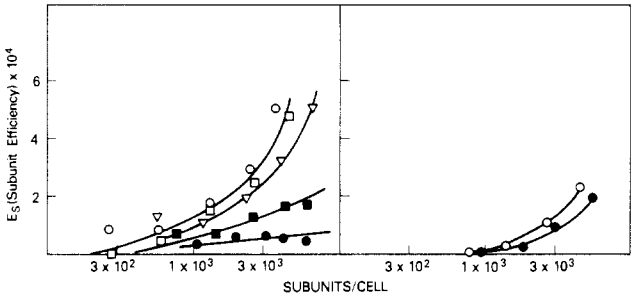

FIGURE 7: Effect of hapten density on lytic efficiencies of monomeric IgG antibodies. Left panel: anti-N_2ph antibodies. Right panel: anti-SRBC antibodies. Sheep erythyrocytes were labeled with (●) 0.21, (■) 0.43, (▽) 0.85, (□) 1.7, and (○) 3.4 mM N_3ph-sulfonic acid.

studies was 50–80 CH_{50} units.

Numbers of Lytic Sites per Cell. The average number of lytic sites per cell (Z) was calculated from the fraction of cells lysed (y) by using the formula $Z = -\ln(1 - y)$ (Mayer, 1961). Values of Z calculated from the data presented in Figure 4B and corrected for antibody-independent lysis (by subtracting values of Z observed in the absence of antibody) are plotted in Figure 6A as a function of the number of bound subunits (N_s). In order to compare the relative efficiencies of oligomers in mediating lysis, each value of Z was divided by the corresponding value of N_s. The derived quotient (Z/N_s) is defined as the "subunit lytic efficiency", E_s, and in Figure 6B values of E_s are plotted as a function of N_s. It is clear from this figure that the lytic efficiencies of constituent IgG subunits increase with oligomer size and that, for each oligomer, efficiency increases with the number of bound subunits.

Subunit efficiencies also increase with complement concentration. Several oligomer preparations, cross-linked with both BDPE and DMS, have been tested over a 16-fold range of complement concentration, and, in every case, the subunit efficiency increased with oligomer size in a manner qualitatively similar to that illustrated in Figure 4B. The DMS oligomers, however, were consistently less efficient at mediating lysis than those cross-linked with the site-specific reagent, BDPE, and efficienices of DMS oligomers were variable between preparations.

A final variable affecting subunit efficiencies was the density of N_3ph on the N_3ph-SRBC. Monomeric anti-N_2ph IgG antibodies showed markedly increased lytic efficiency with increasing concentration of the N_3ph-sulfonic acid used to modify the cells, in the range of 0.21–0.85 mM (Figure 7A). Above 0.85 mM the lytic efficiencies became independent of hapten density. When anti-SRBC IgG antibodies were tested in this system, only a minor change in lytic efficiency was observed with changes in N_3ph density (Figure 7B). Thus, the density effect observed with anti-N_2ph antibodies was hapten specific. Hapten density effects were also observed for BDPE oligomers of all sizes (Figure 8). A monotonic increase in lytic efficiency with oligomer size was observed at all hapten concentrations tested.

The abilities of anti-N_2ph IgG oligomers to mediate lysis were compared with those of anti-N_2ph and anti-SRBC IgM antibodies. In contrast to the results obtained with IgG antibodies and oligomers, lytic efficiencies of the anti-N_2ph IgM preparations were independent of hapten densities on N_3ph-SRBC. The results were variable between preparations as well. In one preparation, for example, the lytic efficiencies of IgM molecules were similar to the efficiencies of IgG heavy oligomers (tetramer–hexamer), while, in another, lytic efficiencies were several-fold higher. Anti-SRBC IgM antibodies were more efficient at mediating lysis than were the anti-N_2ph IgM antibodies and required about six cell-bound molecules for an average of one lytic site per cell, at a complement dilution of 1:6.

Discussion

The data presented in this paper offer direct evidence that clusters of IgG molecules initiate complement-mediated lysis more efficiently than do single IgG molecules. Under all conditions examined in this study, subunit efficiency increased

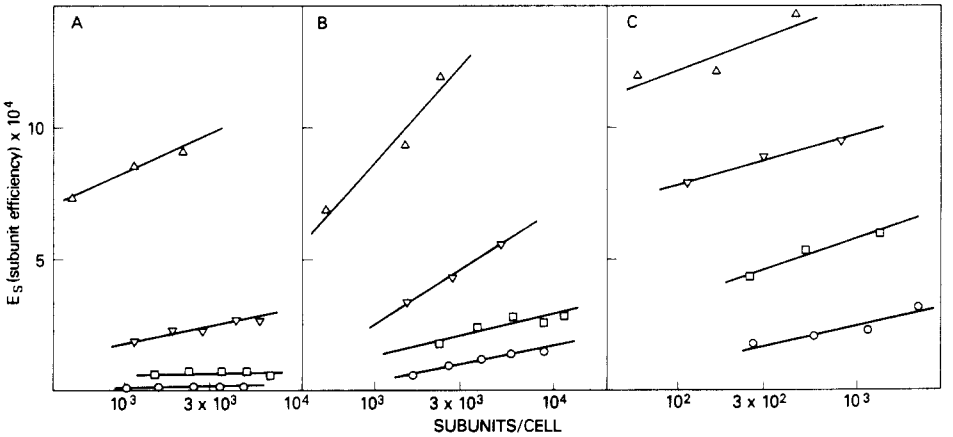

FIGURE 8: Effect of hapten density on lytic efficiencies of BDPE oligomers. The concentrations of N_3ph-sulfonic acid used in making N_3ph-SRBC were (A) 0.23, (B) 0.68, and (C) 3.4 mM. Oligomers are (○) monomer, (□) dimer, (▽) trimer, and (△) a heavier fraction (mostly tetramers, pentamers, and hexamers).

in parallel with oligomer size. Thus, a molecule of monomeric IgG was less efficient at initiating lysis than a molecule of IgG bound in a dimer, and the efficiency increased continuously with oligomer size (Figures 4 and 8). An IgG "unit signal", comparable to the dimer signal of IgE molecules necessary and sufficient for triggering histamine release from mast cells (Segal et al., 1977) could not therefore be demonstrated in this system.

The lytic efficiencies of oligomeric IgG molecules varied with several parameters besides oligomer size. These included the number of subunits bound per cell, the hapten density on target cells, and complement concentration.

(1) As demonstrated by the positive slopes seen in Figures 6B and 8, lytic efficiencies of all oligomers increase with the number of subunits bound per cell. Similar results were obtained at every complement concentration and hapten density tested in this paper. This trend would suggest that clusters of oligomers, which can be presumed to form on cell surfaces at high oligomer density, are more efficient at mediating lysis than single oligomer molecules. The observation that even the largest oligomers (probably hexamers) increased in efficiency with the number of subunits bonds argues against a unit signal model and supports the concept that lytic efficiency increases continuously with cluster size.

(2) As the hapten densities on the target cells increased, lytic efficiencies also increase for all oligomers (Figures 7 and 8). Because the average distance between cell-bound hapten groups would be greater in the lightly substituted cells than in the heavily substituted ones, oligomer molecules would presumably have a higher probability of forming a cluster on a heavily substituted cell than on a cell with low hapten density. Thus, the hapten density effect has a simple explanation consistent with the cluster model for complement lysis. It is of interest that anti-SRBC IgG antibodies are not as efficient at mediating lysis as anti-N_2ph IgG antibodies when bound to heavily substituted N_3ph-SRBC (Figure 7). This suggests that the cell surface density of the sheep erythrocyte antigens might limit the efficiencies of anti-SRBC IgG antibodies.

(3) In all experiments described in this paper, the lytic efficiencies of oligomers increased with increasing complement concentrations. A plateau in the complement concentration dose–response curves could not be found because at high complement concentrations and hapten densities N_3ph-SRBC activate complement by the classical pathway in the absence of antibody (Loos & Thesen, 1978). In other systems not involving N_3ph-SRBC, a plateau is reached (Langone et al., 1978). The relationship between lytic efficiencies of various oligomers was not altered as a function of complement concentration; there was no tendency for the subunit efficiencies of the different-sized oligomers to approach a single value at higher complement concentrations, and large oligomers were always more effective lytic agents than smaller cells.

A major advantage in using the direct lytic system described in this paper is that the interactions of antibodies with relatively high complement concentrations (20-fold dilution) could be studied. Such levels are closer to those found in vivo than the low complement concentrations (1000-fold final dilution) used in the microcomplement fixation assays (Figures 1 and 2) required for measuring the depletion of complement in solution (Wasserman & Levine 1961). The absence of complement fixation by any of the oligomer in unaggregated form (Table I) could well be a result of the low complement levels used in those studies. These same oligomers might well fix complement in solution at higher complement concentrations, since lytic efficiences do increase with complement concentration (Figure 5).

The observations described in this paper are physiologically relevant only if the oligomers are similar to immune complexes formed in nature. Natural immune complexes exist in a large variety of sizes and configurations, and the three types of oligomers used here also presumably exist in a large number of fluctuating configurations. Whether the ranges of configurations are overlapping is not known. Our approach to this problem has been to study oligomers cross-linked in different ways. The observation that the different types of oligomers interact with complement in similar ways suggests that perhaps the mode of cross-linking is not a key factor in determining the lytic efficiencies of oligomers. However, confidence in the assumption that the oligomers are suitable models for immune complexes will increase as other types of oligomers become available and as more comparative analyses of their biological properties are carried out.

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