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# ANTIBODY-DIRECTED LIPOSOMES

## DETERMINATION OF AFFINITY CONSTANTS FOR SOLUBLE AND LIPOSOME-BOUND ANTIFLUORESCEIN

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**We have used the binding of liposomes conjugated with antifluorescein antibody specific for fluorescein isothiocyanate-modified erythrocytes as a model for multivalent antigen-antibody interactions. We examined a series of liposome preparations which were conjugated to between 0 and 332 active antibodies per liposome. The antigen binding capacity and mean intrinsic affinity of the soluble and conjugated antibody were determined by fluorescence quenching of carboxyfluorescein. Liposome-cell interaction data were fitted with a Scatchard-type equation. Functional affinity of liposomes for cells was up to 1000-fold greater than the intrinsic affinity of the antibody for soluble ligand. Analysis for binding at high cell concentrations revealed that liposome-induced cell agglutination reduces the number of available binding sites per cell.**

## Introduction

The implications of monovalent and multivalent antigen-antibody interactions have been discussed by Karush [1], who defined intrinsic affinity as the parameter of monovalent interaction and functional affinity as the parameter of multivalent interaction. Derivation of functional affinity from intrinsic affinity based on theoretical principles is very difficult, because it depends on the number, spatial arrangement and motional freedom of antibody and antigen binding sites [1]. However, it is possible in some circumstances to measure functional affinity empirically. Hornick and Karush [2,3] have determined one functional affinity by measuring the inactivation of dinitrophenylated bacteriophage $T_4$ with anti-dinitrophenol antibodies. They showed the functional affinity of anti-dinitrophenol IgG to be between $2 \cdot 10^4$- and $6 \cdot 10^4$-times greater than its intrinsic affinity measured by equilibrium dialysis [2]. This difference presumably arises from the multivalency of the antibody. Measurement of rate constants for association and dissociation showed that the higher func-

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Abbreviation: FITC, fluorescein isothiocyanate.

tional affinity is due to a lower dissociation rate constant, the association rate constant being unchanged. Hornick and Karush have also shown that the functional affinity of anti-dinitrophenol IgM is much higher than that of an anti-dinitrophenol IgG with a comparable intrinsic affinity for the monovalent antigen, dinitrophenol-lysine [3]. Therefore, an antibody with ten binding sites can bind to a multivalent antigen with greater affinity than an antibody with two binding sites of similar intrinsic affinity.

There has been increasing interest in the covalent attachment of antibodies to the outer surface of liposomes [4], especially as a means of specifically delivering drugs or macromolecules to selected target cells [5]. We have previously described the interaction of antibody-targeted liposomes with erythrocytes and we noted that the amount of liposome-conjugated antibody required to agglutinate erythrocytes was less than the amount of unconjugated antibody needed for agglutination [4]. Since about 300 IgG molecules were attached to each liposome, we speculated that liposome-conjugated antibody was more effective than unconjugated antibody in binding to and agglutinating erythrocytes, because the liposome-cell interaction would be multivalent.

We have noted [5] that the superior efficacy of a multivalent interaction might have important consequences for the use of antibody-targeted liposomes. In addition, this system provides a valuable model for studying the interaction of multivalent receptor complexes with multivalent ligand complexes, a study of considerable importance for lymphocyte-antigen interactions [6]. For these reasons, we have pursued these investigations with immunopurified antifluorescein antibody and erythrocytes conjugated with fluorescein isothiocyanate. The use of anti-fluorescein antibody permits direct and rapid measurement of the intrinsic affinity of the antibody by the fluorescence quenching of fluorescein or its derivatives. Fluorescence quenching is used to determine the antigen-binding capacity of the antibody before and after conjugation, which permits direct measurement of the number of active antigen-binding sites per liposome. The multivalent interaction between the liposomes and the fluoresceinated cells has been examined in binding studies with radiolabelled liposomes. The data have been fit to a theoretical curve which enables us to derive an affinity constant and the number of sites per cell. We find that the affinity of liposomes for the cells is up to 1000-fold greater than the affinity of the antibody for fluorescein. This analysis also provides a quantitative measure for the hemagglutination efficiency of the antibody-coated liposomes.

## Materials and Methods

Phosphatidylcholine was purified as previously described [9]. Cholesterol was purchased from Fluka AG and recrystallized three times from hot methanol. Mixed bovine brain gangliosides were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Immunopurified rabbit anti-fluorescein IgG was prepared as previously described [7] and whole rabbit IgG was purchased from Miles Chemical Co. (Elkhardt, In, U.S.A.). Carboxyfluorescein was obtained from Eastman (Rochester, NY, U.S.A.) and was recrystallized from methanol. Fluorescein isothiocyanate was purchased from Aldrich Chemical Co (St. Louis, MO, U.S.A.). All other materials were reagent grade or better.

Liposomes were prepared in 10 mM acetate/60 mM NaCl (pH 5.5) by the method of Szoka and Papahadjopoulos [8] from phosphatidylcholine/cholesterol/mixed brain ganglioside (40 : 50 : 10) with $2 \cdot 10^{12}$ cpm per mol of [$^3$H]dipalmitoylphosphatidylcholine (spec. act. 500 Ci/mol). Vesicles were centrifuged at $5000 \times g$ for 10 min to remove any multilamellar liposomes and then extruded through 0.4 $\mu$m and 0.2 $\mu$m Bio-Rad Uni-Pore polycarbontate filters [9]. The liposomes were incubated with an equal volume of 10 mM acetate/60 mM periodate in the dark at room temperature for 30 min. The liposomes were separated from excess periodate by chromatography on a $15 \times 1$ cm column of Sephadex G-75. The column was equilibrated with 20 mM borate/120 mM NaCl (pH 8.4). After separation, 0.65 ml of oxidized liposomes (14.5 $\mu$mol lipid/ml) was mixed with 0.1 ml 100 mg/ml IgG solution containing immunopurified anti-fluorescein antibody and normal rabbit IgG in varying proportions. Recrystallized sodium cyanoborohydride was added to 10 mM and the mixture was left overnight to react.

The liposomes were separated on dextran gradients and analyzed for their protein and lipid content as previously described [10]. The activity of the anti-fluorescein antibody was determined by quenching of carboxyfluorescein fluorescence [11]. Successive 20 $\mu$l portions of 200 $\mu$g/ml antibody were added to 1 ml of $1 \cdot 10^{-7}$ M carboxyfluorescein and fluorescence was measured after each addition. Activity was determined by comparing the fluorescence quenching of the unmodified anti-fluorescein to the fluorescence quenching of the liposome-bound anti-fluorescein. Carboxyfluorescein fluorescence was measured in an SLM model 4000 fluorimeter. The excitation wavelength was 493 nm, and fluorescent emission was measured above 520 nm using a Corning 3-68 cut-off filter [12]. Human citrated blood was collected within 2 days of use and stored at 4°C. Prior to use, the erythrocytes were washed five times with phosphate-buffered saline to remove the plasma. Cells were haptenated with fluorescein isothiocyanate by the method of Matuhasi et al. [13]. The extent of fluorescein conjugation was measured by lysing a portion of the erythrocytes and washing the membranes until free of hemoglobin. The fluorescein content of the detergent solubilized ghosts was measured fluorimetrically and was equivalent to $1 \cdot 10^8$ fluorescein isothiocyanate molecules per cell.

Cell-binding studies were performed by incubating $10^6$–$10^8$ erythrocytes with 25–200 nmol lipid for 1 h at 37°C in 0.2 ml phosphate-buffered saline (pH 7.2). Preliminary studies revealed that binding was complete in as little as 5 min at 37°C. The mixture was then layered onto 2 ml 10% (w/v) dextran T40 and centrifuged at 5000 × $g$ for 5 min to pellet the erythrocytes. The supernatant was aspirated, taking care first to remove the interface which contained the unbound liposomes, and the cells were resuspended and rewashed the same way once more. Samples containing $10^8$ cells were lysed and extracted by the method of Bligh and Dyer [14] in order to avoid quenching of scintillation counts by hemoglobin. The chloroform phase was transterred to a glass scintillation vial and evaporated in an oven at 65°C overnight prior to addition of 1 ml water and 10 ml scintillant. Samples containing $10^7$ or $10^6$ cells were taken up directly in scintillant and counted, because these samples contained insufficient hemoglobin to cause quenching. For hapten inhibition, carboxyfluorescein was added at the appropriate concentration to the liposomes before addition of the cells. Incubation and washing of the cells was done as described above except that the first wash contained the hapten at the appropriate concentration. Prior to counting, all samples were extracted by the method of Bligh and Dyer [14] to eliminate quenching by hemoglobin and carboxyfluorescein.

## Results

Coupling of anti-fluorescein antibody to liposomes is summarized in Table I. Conjugation of liposomes with immunopurified anti-fluorescein (conjugate E) gave a final protein : lipid ratio of 166 g/mol. Conjugation to normal rabbit IgG (conjugate A) under identical circumstances gave a protein : lipid ratio of only 82 g/mol, while mixtures of immunopurified anti-fluorescein and normal IgG (conjugates B–D) gave protein : lipid ratios between 82 and 166 g/mol, in accordance with the ratio of anti-fluorescein to normal IgG. This suggests that the anti-fluorescein antibody couples more efficiently to the liposomes than normal rabbit IgG. We have calculated the number of antibody molecules per liposome based on a molecular weight of 150000 for IgG and assuming our preparations contain $8 \cdot 10^{17}$ liposomes per mol lipid (see Appendix, section C). This parameter varies in accord with the antibody : lipid ratio between 410 and 830 molecules per liposome.

Fig. 1 shows the quenching of carboxyfluorescein due to the addition of anti-fluorescein IgG. The IgG was added sequentially in 20 $\mu$l aliquots with 4 $\mu$g ($2.67 \cdot 10^{-11}$ mol) IgG per aliquot to 1 ml of $10^{-7}$ M carboxyfluorescein. The theoretical curves show the expected quenching (including the decrease of fluorescence due to dilution) assuming that the intrinsic binding constant of the carboxyfluorescein to the F(ab) monomer of the IgG is either $2 \cdot 10^6$ $M^{-1}$ or $3.3 \cdot 10^6$ $M^{-1}$. The theoretical analysis used to obtain these curves is derived in section A of the Appendix. From the fit to the data it is evident that the intrinsic binding constant, $K$, is $3 \pm 1 \cdot 10^6$ $M^{-1}$. While there is some indication here that the IgG population is not homogenous, it is clear that the range of

TABLE I

PROPERTIES OF THE ANTIBODY-LIPOSOME CONJUGATES

| Conjugate | Composition of IgG [a] | | Properties of conjugated liposomes | | | |
|---|---|---|---|---|---|---|
| | % anti-FITC | % rabbit | Protein : lipid [b] ($\mu$g/$\mu$mol) | IgG per liposome [c] | Active sites per liposome [d] | % active IgG [e] |
| A | 0 | 100 | 82 | 410 | 0 | 0 |
| B | 10 | 90 | 82 | 410 | 124 | 15 |
| C | 30 | 70 | 105 | 525 | 232 | 22 |
| D | 60 | 40 | 114 | 570 | 400 | 35 |
| E | 100 | 0 | 166 | 830 | 664 | 40 |

[a] These values give the percent of each IgG type used in the conjugation reaction to the liposomes as described in Materials and Methods. Note that the final ratio of anti-FITC to normal rabbit IgG conjugated to the liposomes is not necessarily equal to these ratios.

[b] The ratio of $\mu$g protein conjugated per $\mu$mol lipid was assayed as described in Materials and Methods.

[c] These values are calculated according to the formula: $8 \cdot 10^{11}$ liposomes per $\mu$mol lipid and $4 \cdot 10^{12}$ IgG per $\mu$g ($1.5 \cdot 10^5$ g IgG/mol).

[d] These values are obtained by the fitted data as described in Fig. 2.

[e] Note that each IgG has two sites, although either or both may be inactive.

binding constants is covered by the uncertainty given above for the value of $K$. Overall, the possible heterogeneity has a very minor effect.

The activity of the liposome-bound antibody was assessed by fluorescence quenching of carboxyfluorescein. Quench data were fitted by Eqn. 9 in the Appendix to determine the antigen-binding capacity of the liposome-conjugated antibody assuming that the monovalent affinity constant remained at $3 \cdot 10^6$ $M^{-1}$. The curves for conjugates A, C and E are shown in Fig. 2, and the activity of all conjugates is summarized in Table I. The number of active anti-fluorescein sites per liposome ranges from 124 for conjugate B to 664 for conjugate E. Conjugate E retained 40% of the antigen-binding capacity of the original antibody,

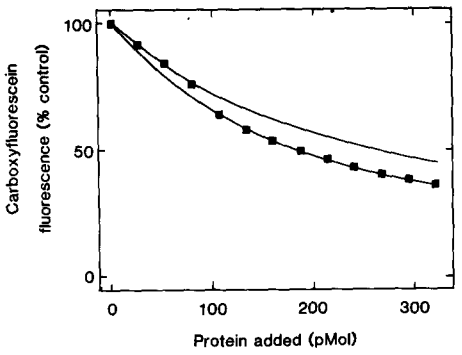


Fig. 1. Quenching of carboxyfluorescein fluorescence due to the addition of immunopurified soluble anti-fluorescein IgG. Successive 20 $\mu$l aliquots of IgG (26.7 pmol protein in each) are added to 1 ml (initially) of $1 \cdot 10^{-7}$ M carboxyfluorescein. The binding of the carboxyfluorescein to each active IgG site quenches 90% of its fluorescence [11]. The loss in fluorescence is due to this binding plus dilution. The theoretical curves are calculated from Eqns. 9 and 10 assuming two active sites per IgG. The upper curve assumes a binding constant, $K$, $2 \cdot 10^6$ $M^{-1}$, whereas the lower curve assumes a binding constant, $K$, of $3.3 \cdot 10^6$ $M^{-1}$.

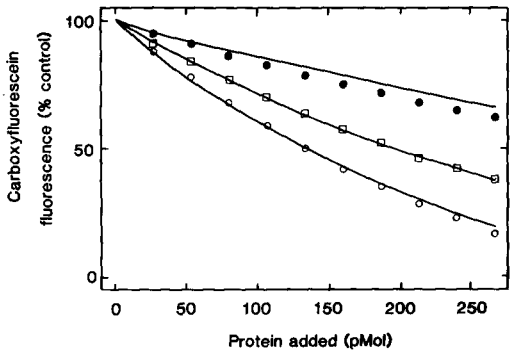


Fig. 2. Quenching of carboxyfluorescein by IgG conjugated to liposomes: conjugate A (●), conjugate E (○) and conjugate C (□) (see Table I). The theoretical curves were fitted using Eqns. 9 and 12, to account for dilution, assuming that the binding constant of carboxyfluorescein to each active anti-fluorescein IgG active site, $K$, is $K = 3 \cdot 10^6$ $M^{-1}$. For conjugate A, 100% normal rabbit IgG, the theoretical curve shows the effect of dilution only, i.e., no quenching due to specific binding is assumed. The data points show a small but insignificant degree of quenching.

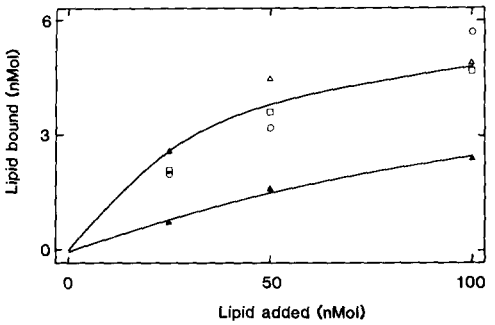


Fig. 3. The binding of anti-fluorescein conjugated liposomes to FITC-conjugated erythrocytes. The different liposomes conjugates (B (▲), C (□), D (△) and E (○)) were incubated with $10^6$ erythrocytes in 0.2 ml as described in Materials and Methods. The theoretical curves were fitted assuming 5000 liposome binding sites per cell, which is the maximum number assuming close packing, and a liposome binding avidity of $K_a = 1 \cdot 10^9$ $M^{-1}$ (lower curve) for conjugate B and $K_a = 5 \cdot 10^9$ $M^{-1}$ (upper curve) for conjugates C–E according to Eqn. 17 in the Appendix. Conjugate A, the control, showed less than 0.2 nmol lipid bound over the entire concentration range (not shown).

which shows that inactivation of the antibody occurs during covalent attachment. If we assume that anti-fluorescein is always 60% inactivated upon conjugation, conjugates B–D show higher levels of activity than expected from the initial ratios of normal IgG and anti-fluorescein IgG. This further confirms the more efficient conjugation of anti-fluorescein antibody to the liposomes.

*Cell binding*

Fig. 3 shows the amounts of lipid bound to $10^6$ erythrocytes as a function of the added lipid for the conjugated liposomes described in Table I. The data for conjugates C–E is essentially identical, showing that the binding is insensitive to the density of anti-fluorescein once there are more than 230 active sites per liposome. However, conjugate B (124 anti-FITC sites per liposome) shows a diminished binding capacity. In order to fit our data we need to make estimates of the mean affinity and the number of binding sites per cell. We have estimated the maximum number of binding sites per cell by determining how many liposomes can be closely packed on the surface of the erythrocyte (see Appendix). This value is approx. 5000 sites per cell. If we assume that there are 5000 sites per cell, conjugates C–E must have a binding avidity, $K_a$, of $5 \cdot 10^9$ $M^{-1}$; whereas, conjugate B must have a $K_a$ of $1 \cdot 10^9$ $M^{-1}$.

Fig. 4 shows the binding results with $10^7$ erythrocytes. The theoretical curves are calculated from Eqn. 17 (see Appendix) using 5000 sites per cell with the appropriate $K_a$ values derived from Fig. 3. The data for conjugate B fit well but the fit for conjugates C–E is not adequate at the lower lipid concentrations. In order to fit these datum points, we must either assume an apparent reduction in mean avidity (which would make little physical sense) or a reduction in the number of sites per cell. Visual inspection of the cell binding experiments revealed agglutination when $10^7$ cells were present, but not when $10^6$ cells were present. We believe that cell agglutination will occlude some of the cells' surfaces, which will reduce the number of available binding sites if it occurs before binding is complete. This effect would be most apparent at the low liposome concentrations, where the kinetics of liposome-cell binding would be slow, and with high erythrocyte concentrations, where the agglutination reaction would be rapid [15,16].

Fig. 5A shows the binding of conjugate B to $10^8$ cells. The upper theoretical curve presumes 5000 available sites per cell and the lower curve presumes 3000 available sites per cell. Both curves assume a binding avidity of $1 \cdot 10^9$ $M^{-1}$. The effect of agglutination is seen here, although it does not substantially reduce the liposome bind-

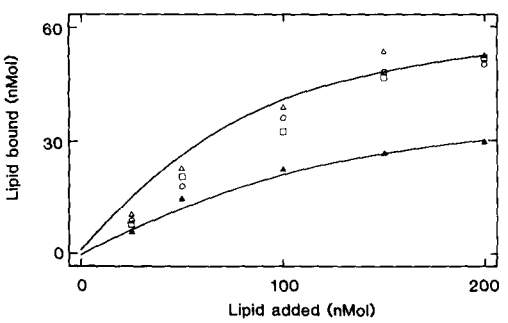


Fig. 4. Binding of anti-fluorescein conjugated liposomes to $10^7$ FITC-conjugated erythrocytes. The symbols are the same as in Fig. 3. The theoretical curves are obtained as described in Fig. 3, where the lower curve assumes $K_a = 1 \cdot 10^9$ $M^{-1}$ and the upper curve assumes $K_a = 5 \cdot 10^9$ $M^{-1}$.

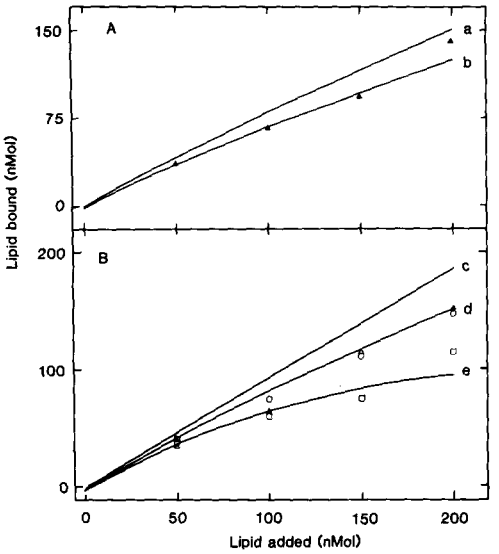


Fig. 5. Binding of anti-fluorescein-conjugated liposomes to $10^8$ FITC-conjugated erythrocytes. The symbols are the same as those in Fig. 3. Conjugate B is shown in panel A, where the theoretical binding curves for 5000 (curve a) and 3000 (curve b) sites per cell are given. Conjugates C–D are shown in part (B) with the theoretical curves for 5000 (curve c), 2000 (curve d) and 1000 (curve e) sites per cell.

ing. An excess of binding sites remains, because the maximum amount of lipid which could be bound to $10^8$ erythrocytes with 5000 sites each is 625 nmol.

The binding of conjugates C–E to $10^8$ erythrocytes is shown in Fig. 5B. These curves are best fit by assuming only 1000–2000 available sites per cell for reversible binding. This clearly shows the effect of agglutination on the available number of binding sites. The curve for 5000 sites per cell is also shown for comparison.

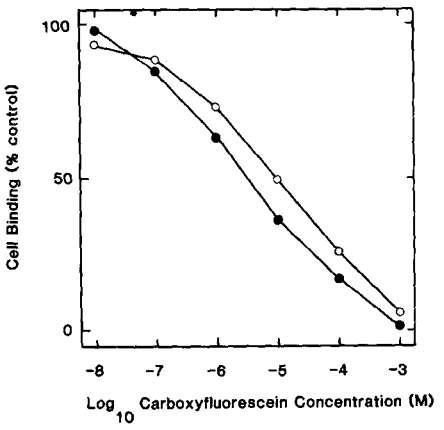


Fig. 6. The inhibition of vesicle-cell interaction by soluble carboxyfluorescein. $10^8$ (○) or $10^7$ (●) FITC-conjugated erythrocytes were incubated with 50 nmol of liposomes (conjugates E) in 0.2 ml phosphate-buffered saline. Cell binding is expressed as a percentage of the binding in the absence of carboxyfluorescein.

TABLE II

INHIBITION OF VESICLE BINDING TO CELLS BY $10^{-6}$ M CARBOXYFLUORESCEIN

See Table I for description of conjugates. Cells were mixed with $10^{-6}$ M carboxyfluorescein prior to the addition of 50 nmol lipid. Binding is expressed as a percent of binding in the absence of carboxyfluorescein (see Figs. 4 and 5 for control levels).

| Conjugate | Vesicle binding to cells (% control) | |
|---|---|---|
| | $10^8$ cells | $10^7$ cells |
| E | 76 | 66 |
| C | 68 | 23 |
| B | 54 | 9 |

*Hapten inhibition*

Fig. 6 shows the binding of 50 nmol of conjugate E to $10^8$ or $10^7$ erythrocytes in the presence of carboxyfluorescein. Binding inhibition is detectable at concentrations as low as $10^{-7}$ M carboxyfluorescein and $10^{-3}$ M carboxyfluorescein eliminates all specific binding. This further demonstrates the specificity of the targeted liposomes for binding to fluorescein ligand. The inhibition of binding appears, for a given carboxyfluorescein concentration, to be slightly greater for the samples containing $10^7$ cells than for the samples containing $10^8$ cells. Table II shows the inhibition of binding of all the conjugates by $10^{-6}$ M carboxyfluorescein. For the incubations containing $10^7$ cells, there are pronounced differences in the proportion of the binding of the different conjugates which is inhibited by this concentration of soluble hapten. This suggests that the number of antibody molecules per liposome affects the ligand inhibition of binding of the liposomes to the cell surface. This result is consistent with cell binding studies described above.

## Discussion

The analysis of the binding of carboxyfluorescein to the IgG as soluble protein (Fig. 1)

and conjugated to liposomes (Fig. 2) is based on a simple mass action equilibrium. We presumed that the soluble IgG could bind two carboxyfluoresceins [11], hence the concentration of binding sites was twice the IgG concentration. With these assumptions, we have calculated an intrinsic affinity of $(3 \pm 1) \cdot 10^6$ $M^{-1}$. If only one site were active, the intrinsic affinity constant would be $6 \cdot 10^6$ $M^{-1}$. The concentrations of IgG and carboxyfluorescein used to measure antifluorescein activity would not allow us to detect sites whose intrinsic affinity constant was less than one-tenth the mean. Therefore, we can disregard the possible effects of the heterogeneity of the IgG binding sites, which would be apparent only at very high carboxyfluorescein concentrations.

For the IgG conjugated to liposomes, we report the number of active sites per liposome using this binding constant for each active site. Conjugate E, which is conjugated with pure anti-fluorescein, suffers about 60% inactivation. Moreover, anti-fluorescein appears to conjugate to liposomes more readily than normal IgG. These two observations suggest that conjugation may block one active site of anti-fluorescein IgG. Consequently, the liposomes may present an array of active monomeric binding sites for the carboxyfluorescein and for the fluorescein isothiocyanate (FITC) conjugated to the erythrocytes.

The binding of the liposomes to the erythrocytes is also modelled as a simple mass action equilibrium, where the data are fitted from an equation equivalent to a Scatchard plot (Eqn. 17, Appendix). For any system, the two parameters are the functional affinity of the liposome for a site, $K_a$, and the number of binding sites per erythrocyte, $S_0$. We determined that there should be 5000 liposome binding sites per cell based on a simple steric calculation. This assumption requires that liposomes be able to interact with the entire erythrocyte surface. We believe that this assumption is justified, since there are $1 \cdot 10^8$ FITC groups per cell, and each liposome may interact via a few IgG molecules. Furthermore, this assumption was verified by the data for $10^6$ erythrocytes (Fig. 3), where a Scatchard plot of the combined data for conjugates C–E produced best estimates (least-squares regression of Eqn. 16) of $3.5 \cdot 10^9$ $M^{-1}$ for $K_a$ and 5500 for $S_0$. Similar treatment of the data for conjugate B gave values of $1.1 \cdot 10^9$ $M^{-1}$ for $K_a$ and 4300 for $S_0$. The regression of the data for conjugate B, while of limited statistical significance, lend weight to our assumption that it binds less than the other conjugates because it has a smaller avidity and not because it sees fewer sites on the cell. Relative to the IgG monomers, for which $K = 3 \cdot 10^6$ $M^{-1}$, the liposomes show a 1000-fold greater binding constant for conjugates C–E and a 300-fold greater constant for conjugate B.

The fit to the data for $10^7$ erythrocytes (Fig. 4) using the binding avidities derived from Fig. 3 and assuming 5000 sites per cell is good. However, at the lower lipid concentrations there is somewhat less lipid bound than predicted. We believe that this effect is due to hemagglutination. The lower amounts of bound lipid must be due to either a lowering of the binding avidity or the loss of sites available for reversible binding. While it is difficult to imagine how hemagglutination could reduce the binding avidity, it is easy to understand how it could reduce the number of binding sites. A few bound liposomes could bridge between two erythrocytes, thereby bringing the cells' surfaces too close together to allow other liposomes access to the apposed surfaces. The cells may agglutinate as rouleaux, which would prevent the binding of liposomes to the concave surfaces of the erythrocytes. The extent to which hemagglutination occludes potential binding sites would depend on the relative kinetics of liposome binding versus hemagglutination. The maximal effect would occur when there are few liposomes bound, only enough to hemagglutinate the cells, and when there are many erythrocytes so that the cells have high collision rates [15,16].

This is precisely what we see in cell-binding experiments involving $10^8$ erythrocytes (Fig. 5) where the fit to the data, assuming the same avidity constants as used before, allows only approx. 3000 accessible sites per cell for conjugate B. Moreover, the best fit for conjugates C–E assumes only 1000–2000 accessible sites per cell. The effect of this large reduction in the number of available sites is not very dramatic, since the amount of bound lipid is reduced by only 20–30%. This arises because there is still an excess of available sites due to the large number of cells.

This analysis has allowed us to quantitate the effect of multivalency on IgG-antigen interactions. However, we cannot estimate what proportion of the liposome-conjugated IgGs are bound to the cell. In the simplest viewpoint the effect of having two active sites bound, rather than one, would yield a binding constant of $K^2$, where $K$ is the monomer binding constant. This assumes that both sites can optimally fit into the antigenic binding sites. The reason for the liposomes' not having binding avidities exceeding $10^{12}$ $M^{-1}$ is quite likely due to substantially greater off-rates of the IgGs bound to the liposomes. Other factors, such as the slower on-rates due to the larger size of the liposome and the possibility that the IgGs are sterically hindered from the optimal binding positions, all contribute to lowering the binding avidity from the theoretical maximum. Nevertheless, it is clear that the measured binding avidities, $3 \cdot 10^9$ $M^{-1}$ for conjugates C–E and $1 \cdot 10^9$ $M^{-1}$ for conjugate B, represent a substantial enhancement due to the multivalency. The fact that conjugates C–E all have the same binding avidity shows that the surface density of more than 230 active sites per liposome gives a maximal effect. Conjugate B, with 124 active sites per liposome has a binding constant which is 3-times smaller than that of conjugates C–E. Since the liposome can present only approx. 1/3 of its surface to the erythrocyte surface, it would appear that the maximal interaction occurs with approx. 75 active sites on the apposed surface of the liposome, whereas reducing the number of accessible sites to 40 yields a 3-fold decrease in the binding avidity. Clearly, the effect of these surface densities of active sites depends upon the high density of FITC conjugated to the erythrocyte. At the level of substitution used in these experiments there is approx. one FITC per 150 Å$^2$, assuming a homogeneous distribution. This is equivalent to having approx. 20000 FITC per liposome binding site, although many of the FITC groups may be conjugated to regions inaccessible to the IgG on the liposome.

In conclusion, the functional affinity of an antibody-directed liposome for the surface of an erythrocyte is $10^3$-times the intrinsic affinity of the antigen binding site of the antibody. This may have important consequences for liposome-cell interactions, and further investigation can elucidate how antigen density, antibody density and particle size affect the functional affinity of multivalent complexes.

## Appendix

*Analysis of results*

Anti-FITC IgG has a maximum of two sites per molecule capable of binding either carboxyfluorescein or FITC conjugated to the erythrocytes. However, the conjugation of the IgG to the liposome can inactivate some of these sites. Hence, a given IgG molecule will have two, one or zero active binding sites. We assume that all active binding sites are identical and equivalent with an intrinsic binding constant, $K$. Likewise, all inactive binding sites are assumed to have an intrinsic binding constant of zero; however, we note here that the binding constant need be reduced only by an order of magnitude for the site to be negligible in these experiments.

We will develop the analysis used to fit the data here. The equations for the carboxyfluorescein quenching will be obtained explicitly for IgG with two active sites; however, since we assume that the binding sites are equivalent and independent, the final equation (Eqns. 9 and 12) are appropriate for IgG conjugated to liposomes with some inactivated sites.

*A. Carboxyfluorescein quenching*

The binding of carboxyfluorescein to the anti-FITC IgG (either soluble or conjugated to liposomes) follows the reactions

$$\begin{aligned} F + P &\overset{K_1}{\leftrightharpoons} P_1 \\ F + P_1 &\overset{K_2}{\leftrightharpoons} P_2 \end{aligned} \qquad (1)$$

where F denotes carboxyfluorescein, P denotes the unbound IgG and $P_1$ and $P_2$ denote the IgG with one or two bound carboxyfluorescein molecules. Clearly, if the IgG has only one active site, then it follows only the first reaction. When $K$ denotes the intrinsic binding constant to the F(ab) monomer, and there are two active F(ab) monomers per IgG,

then

$$K_1 = 2K$$
$$K_2 = \tfrac{1}{2}K \tag{2}$$

due to the statistics of equivalent and identical binding sites [17]. For those IgG molecules with only one active site, $K_1 = K$ by definition.

Since

$$K_1 = [P_1]/[F][P]$$
$$K_2 = [P_2]/[F][P_1] \tag{3}$$

where the brackets denote molar concentrations, we can write the conservation relations for the total IgG concentration, $[P_t]$, and the total carboxyfluorescein concentration, $[F_t]$ as

$$\begin{aligned}[P_t] &= [P]+[P_1]+[P_2] \\ &= [P]+K_1[F][P]+K_2[F][P_1] \\ &= [P]\left(1+2K[F]+K^2[F]^2\right) \\ &= [P](1+K[F])^2 \end{aligned} \tag{4}$$

using Eqns. 2–3, and

$$\begin{aligned}[F_t] &= [F]+[P_1]+2[P_2] \\ &= [F]+2[P]K[F](1+K[F]) \\ &= [F]+2[P_t]K[F]/(1+K[F]) \end{aligned} \tag{5}$$

using Eqns. 2–4.

We use the same procedure for the general case where some of the added IgG has inactive binding sites. Let $[P_{it}]$ denote the total concentration of IgG which has $i$ active binding sites and $2-i$ inactive binding sites. Then the total IgG concentration is

$$[P_t] = [P_{2t}]+[P_{1t}]+[P_{0t}] \tag{6}$$

and the average number of active binding sites per IgG is

$$\rho = (2[P_{2t}]+[P_{1t}])/[P_t] \tag{7}$$

Thus the conservation relation for the total carboxyfluorescein concentration is written as

$$\begin{aligned}[F_t] &= [F]+\frac{K[F]}{1+K[F]}(2[P_{2t}]+[P_{1t}]) \\ &= [F]+\rho[P_t]\frac{K[F]}{1+K[F]} \end{aligned} \tag{8}$$

using Eqns. 6–7. Note that when all binding sites are active, then $\rho = 2$ and Eqn. 5 is recovered. If all IgG have only one active sites, then $\rho = 1$ and the simple 1-1 binding equation is obtained. Solving Eqn. 8 for the free carboxyfluorescein concentration yields

$$[F] = \frac{1}{2K}\left\{K([F_t]-\rho[P_t])-1 + \sqrt{(K(\rho[P_t]-[F_t])+1)^2+4K[F_t]}\right\} \tag{9}$$

We note again that this equation is completely valid for IgG conjugated to liposomes, even if the liposome is considered to be a polyvalent species, as long as the binding sites are equivalent and independent. The values of $\rho$ found for the liposome conjugated IgG are given in Table I.

Since the carboxyfluorescein quenching data were obtained by a sequential titration of the IgG, which would decrease the fluorescence both by specific binding and dilution, it is expedient here to show the equations used to fit the data of Fig. 1 and Table I. Let $[F_t]_0$ denote the carboxyfluorescein concentration initially in the 1 ml cuvette. Each aliquot of $\nu$ ml contains $\delta$ mmol of IgG (either soluble or conjugated to liposomes). Thus, after $n$ aliquots, the volume of solution in the cuvette is $(1 + n\nu)$ ml. The IgG concentration is

$$[P_a] = n\delta/(1+n\nu) \tag{10}$$

and the total carboxyfluorescein concentration is

$$[F_t] = [F_t]_0/(1+n\nu) \tag{11}$$

The initial fluorescence intensity for $[F_t]_0$, is set to 100% and the relative fluorescence after $n$ aliquots is

$$I = 100([F]+0.1[F_b])/[F_t]_0 \tag{12}$$

where $[F_b]$ denotes the concentration of bound carboxyfluorescein, i.e., $[F_b] = [F_t] - [F]$. The factor 0.1 implies that the bound carboxyfluorescein still retains 10% of its fluorescence [11].

Notice that if there were no binding, then $[F_b] = 0$, $[F] = [F_t] = [F_t]_0/(1 + n\nu)$ and $I = 100/(1 + n\nu)$, which shows the decrease in fluorescence due only to dilution. If all of the carboxyfluorescein were bound, then $[F] = 0$, $[F_b] = [F_t]$ and $I = 10/(1 + n\nu)$, which reflects the residual 10% fluorescence of the bound molecule, together with the dilution factor.

*B. Binding of liposomes to erythrocytes*

The liposome binds to the cell according to the reaction

$$X + S \overset{K_a}{\leftrightharpoons} XS \tag{13}$$

where X denotes the liposome, S denotes the site on the cells' surface and XS denotes the bound liposome or the occupied site. The binding constant is defined by

$$K_a = [XS]/[X][S] \tag{14}$$

where [X] is the bulk molar concentration of liposomes and [XS] and [S] denote the surface densities of occupied and unoccupied sites on the erythrocyte's surface. The binding constant, $K_a$, represents a crude estimate for the extent to which the liposome binding is governed by multivalent bridging. However, the extent to which $K_a$ exceeds the intrinsic binding constant $K$ for the F(ab) monomer to the fluorescein does prove the existence of the liposome's multivalent binding.

This formulation of the binding reaction assumes that each of the cell's binding sites is equivalent and independent. However, as we show, agglutination of the erythrocytes by the liposomes causes the occlusion of some binding sites, hence the total number of sites available to this equilibrium binding can be reduced. We account for this fact in the simplest way, i.e., to fit the value of $K_a$ by cases where the agglutination is negligible (Fig. 2) and to fit the total number of available binding sites, in those cases where agglutination is significant using the known value of $K_a$.

When $[S_0]$ denotes the total (average) number of available binding sites per cell and $[X_0]$ denotes the initial concentration of liposomes, we can write the conservation relation

$$[S_0] = [S] + [XS]$$

$$[X_0] = [X] + [C][XS] \tag{15}$$

where [C] is the concentration of erythrocytes. Combining Eqns. 14 and 15 leads to the following equation for the concentration of bound liposomes $[X_b] = [C][XS]$ as

$$\frac{[X_0]}{[X_b]} = \frac{[X_0] - [X_b]}{[C][S_0]} + 1 + \frac{1}{K_a[C][S_0]} \tag{16}$$

Hence the linear regression of $[X_0]/[X_b]$ vs. $[X_0] - [X_b]$ will yield $1/[C][S_0]$ for a slope and $1 + (1/K_a[C][S_0])$ for an intercept; hence, both $[S_0]$ and $K_a$ may be estimated. Eqn. 16 is formally equivalent to a Scatchard plot. For fitting the data in Figs. 2–4, we solved Eqn. 16 for $[X_b]$ as

$$[X_b] = \frac{1}{2K_a}\left\{1 + K_a([C][S_0] + [X_0]) - \sqrt{(1 + K_a([C][S_0] + [X_0]))^2 - 4K_a^2[C][S_0][X_0]}\right\} \tag{17}$$

*C. The maximum number of binding sites per cell*

Preliminary analysis of the data showed in those cases where hemagglutination was not occluding binding sites the number of binding sites per erythrocyte, $[S_0]$ to be approx. 5000. Szoka et al. [9] have shown for a similar lipid mixture (phosphatidylglycerol/ PC/ cholesterol, 1 : 4 : 5) extruded through a 0.2 $\mu$m polycarbonate filter, as was done here, that the mean liposome diameter is 0.17–0.19 $\mu$m. A simple geometric argument shows that the close packing of spheres of diameter, $d$, on a surface of area $A$ can accommodate only $[S_0] = 2A/d^2\sqrt{3}$ spheres. Simply dividing the area, $A$, by the cross-sectional area of the sphere, i.e., $4A/\pi d^2$, neglects the space between the spheres, although this amounts to only a 10% error. Taking 150 $\mu m^2$ as the average surface area of the erythrocyte and 0.18 $\mu$m as the average diameter of the liposomes yields $S_0^{max} = 5346$. Thus, this rough calculation

makes it clear that the entire surface of the erythrocyte is available for specific interaction with the anti-FITC IgG conjugated to the liposomes. The density of FITC conjugated to the erythrocyte sufficiently high that the limiting factors to binding are the surface area available after agglutination and the surface density of IgG on the liposomes. The latter property will manifest itself in the value of $K_a$ which depends on the multivalency of the binding. For the purposes of fitting the data, we have fixed $[S_0]$ at 5000 sites per erythrocyte except in those cases where agglutination reduced the surface area available for reversible binding.

The other physical parameter we require is the number of liposomes per nmol of lipid. The 1 : 1 PC/cholesterol mixture gives a surface area of $2.6 \cdot 10^8$ $\mu m^2$ per nmol lipid [9]. Martin et al. [18] found that these liposomes have only about one-third of their total lipid on the exterior monolayer, due to some liposomes being oligomeric. Hence, using 0.18 $\mu$m for the diameter, we find that the average liposome has 0.3 $\mu m^2$ of surface area, i.e., there are $8 \cdot 10^8$ liposomes per nmol of lipid.

The liposome to cell binding data are presented in terms of nmol of lipid initially added, $L_0$, and bound, $L_b$, to the erythrocytes. To facilitate use of the binding equations with data of this form, we note that the sample volume is 0.2 ml; hence, adding $L_0$ nmol lipid gives an initial concentration of liposomes $[X_0] = L_0$ $(8 \cdot 10^8/0.2)$ liposomes/ml $= (L_0/1.5 \cdot 10^{11})$ M (liposomes). Likewise, $L_b$ nmol lipid bound implies that $[X_b] \doteq L_b(1.5 \cdot 10^{11})$ M (liposomes). When $C_0$ denotes the number of erythrocytes added to 0.2 ml, the concentration of cells [C] is $C_0/(1.2 \cdot 10^{20})$ M (cells).

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