RECEPTORS ON IMMUNOCOMPETENT CELLS

IV. Direct Measurement of Avidity of Cell Receptors and Cooperative Binding of Multivalent Ligands

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Antigen may be demonstrated by radioautography or immunofluorescence to bind to the surface of individual small lymphocytes. Antigen-binding cells (ABC),[1] specific for any given antigen, are rare in nonimmunized animals and increase in frequency after immunization. These cells possess easily detectable amounts of surface immunoglobulin and the blocking of this immunoglobulin inhibits antigen binding (1–5). Specific destruction or removal of ABC of a given specificity reduces the ability of the remaining cells to adoptively transfer primary or secondary antibody responsivenesss to that antigen to an irradiated recipient (4, 6, 7). These ABC appear to be members of the "bone marrow–derived" lymphocyte class and to include the precursors of antibody-synthesizing cells. The antigen-binding surface immunoglobulin molecules are, very likely, the antigen-binding receptors of this class of immunocompetent cells.

In this paper we describe methods whereby the binding of radiolabeled antigen to cell surface receptors can be measured in thermodynamic terms and we present the application of these methods to the binding of ^{125}I-labeled 2,4-dinitrophenyl guinea pig albumin (DNP-GPA) to lymphocytes obtained from guinea pigs immunized with DNP-GPA. We show that the binding of DNP-GPA–^{125}I is a reversible, temperature-dependent process. Association and dissociation rates have been determined and cell-antigen equilibrium constants estimated both from rate constants and from equilibrium-binding data. Those constants change with time after immunization, as does the average intrinsic association constant for hapten of serum antibody molecules. Furthermore, the avidity of ABC for multivalent DNP ligands is much higher than for univalent ligands. This directly establishes that multivalent binding is energetically advantageous in the binding of antigen to immunocompetent cells and provides an estimate of the magnitude of this potentially important biological effect.

[1] *Abbreviations used in this paper:* ABC, antigen-binding cell(s); B cells, bone marrow–derived cells; BF, bovine fibrinogen; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenyl; GPA, guinea pig albumin; KLH, keyhole limpet hemocyanin; T cells, thymus-derived cells.

Materials and Methods

Immunization Protocol and Preparation of Cells.—Adult strain 13 guinea pigs, obtained from the Division of Research Services of the National Institutes of Health, were immunized by injection, into the four footpads, of 50 μg of $DNP_{16}GPA$ emulsified in 0.4 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). At various times after immunization, the animals were exsanguinated and the draining lymph nodes were removed. Cell suspensions were prepared by teasing the lymph nodes with forceps in minimum essential medium (Grand Island Biological Co., Rockville, Md.) containing 10% fetal calf serum. The suspensions were passed through sterile gauze pads to remove cell aggregates and the cells were then washed three times in medium.

Antigens.—Guinea pig albumin (GPA) was prepared by the method of Schwert (8) from strain 13 guinea pig serum; bovine fibrinogen (BF) was obtained from Mann Research Labs. Inc. (New York); keyhole limpet hemocyanin (KLH) was obtained from Pacific Biomarine Supply Co. (Venice, Calif.); bovine serum albumin (BSA) was obtained from Miles Laboratories, Inc. (Elkhart, Ind.). Dinitrophenyl conjugates of these proteins were prepared using 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals, Rochester, N.Y.) (9). The degree of conjugation of each antigen is indicated by subscripts. The subscripts of all antigens except for DNP-KLH refer to moles of DNP per mole of protein utilizing the molecular weights of the native proteins for this calculation. These molecular weights are: GPA, 69,000; BSA, 69,000; BF, 330,000. The subscript for DNP-KLH refers to moles of DNP per 100,000 grams of KLH.

Radioiodinated antigens were prepared by the chloramine-T method (10). Specific activities were as follows: $DNP_{16}GPA$-^{125}I, 20 μCi/μg; DNP_4BSA-^{125}I, 59 μCi/μg; and DNP_9KLH-^{125}I, 21 μCi/μg.

Sucrose density gradient centrifugation of DNP_9KLH showed that the antigen existed in two major forms: 37% of the material had a molecular weight greater than 1×10^6 daltons the remainder had a molecular weight of about 0.5×10^6 daltons. $DNP_{109}BF$ existed only as aggregates of greater than 1×10^6 daltons.

Antigen-Binding Assay.—The details of the antigen-binding method have been presented previously (4, 5). Briefly, 10–30 $\times 10^6$ lymph node cells were suspended in 0.2 ml of minimum essential medium containing 10% fetal calf serum and 1 mg/ml sodium azide. ^{125}I-labeled antigen was added to the cell suspension, generally to a final concentration of 200–250 ng/ml. After a 30 min incubation at 4°C the cells were transferred in 1 ml of fresh medium and layered over 3 ml of fetal calf serum in a 17 x 100 mm plastic culture tube (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.). The cells were sedimented through the fetal calf serum by centrifugation in a swinging bucket centrifuge at 1000 rpm for 10 min. This washing method allows more efficient removal of unbound radioiodinated antigen than more usual methods which rely on dilution. The sedimentation through fetal calf serum was repeated a total of four times. The radioactivity of the final washed pellet was then measured in a γ-ray spectrometer to determine the total amount of antigen bound to the cells. In certain experiments, the amount of antigen, the time of exposure, or the temperature of exposure was varied. Each assay was performed at least in triplicate. Initial studies in which nine replicate samples were measured showed standard errors of $\pm$1–3%.

Enumeration of ABC.—Individual lymphocytes which bound $DNP_{16}GPA$-^{125}I to their surface were detected by radioautography, as previously described (4). In brief, cells from the cell pellet prepared above were smeared on a slide, dipped in Kodak NTB-2 emulsion, and exposed for 1–5 days. Cells with 3 or more grains directly over their surface were classed as antigen binding.

RESULTS

Concentration Dependence of $DNP_{16}GPA$-^{125}I Binding to Lymphocyte Receptors.—Previous studies from this laboratory have demonstrated that lymphoid cells obtained from guinea

pigs immunized with DNP-GPA bind sufficient ligand-^{125}I so that they can easily be detected by measuring the radioactivity of the cell pellet. Moreover, the amount of antigen bound by a cell population correlates well with the number of antigen-binding lymphocytes in the population. Thus, prior treatment of the cells with anti-immunoglobulin serum causes a similar diminution in the amount of $DNP_{16}GPA$-^{125}I which the cells bind and in the number of cells which can be demonstrated to bind $DNP_{16}GPA$-^{125}I by radioautographic methods. In addition, the capacity of an individual cell or of a cell population to bind $DNP_{16}GPA$-^{125}I appears to depend on a product of the binding cell; incubation of cells from nonimmunized animals with large amounts of anti-DNP antibody, followed by washing, does not significantly increase the total number of cells which can bind antigen nor the total amount of antigen bound by the entire population (5).

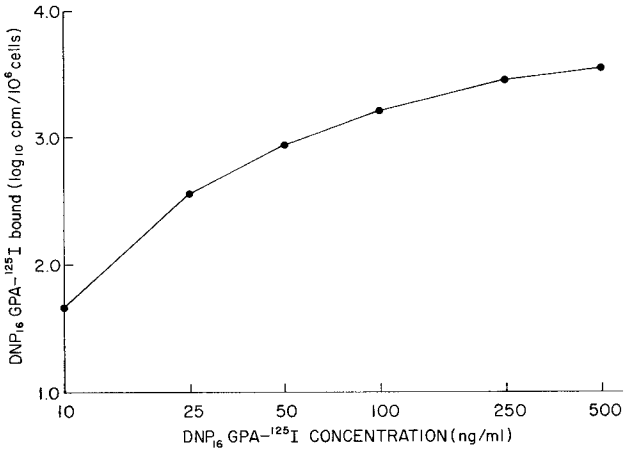

FIG. 1. Effect of $DNP_{16}GPA$-^{125}I concentration on the amount of antigen bound to immune lymphocytes. 18×10^6 lymph node cells from a guinea pig immunized 35 days previously with $DNP_{16}GPA$ were incubated with various concentrations of $DNP_{16}GPA$-^{125}I under conditions described in Materials and Methods. After unbound antigen was removed by centrifugation of cells through fetal calf serum, the amount of antigen adsorbed to the cells was determined.

If the antigen-binding ability of small lymphocytes indeed rests with specific cell-associated antibody-like receptors, then the binding kinetics should have certain predictable properties. First, the binding should be concentration dependent and, with the use of sufficient ligand, saturation should be attained. Lymph node cells from a guinea pig immunized 35 days previously with $DNP_{16}GPA$ were incubated with various amounts of $DNP_{16}GPA$-^{125}I for 30 min at 4°C. After four washes through fetal calf serum, the amount of antigen adsorbed to the cells was determined by measurement of the radioactivity associated with the washed cells.

It is seen in Fig. 1 that the amount of $DNP_{16}GPA$-^{125}I bound by lymphoid cells increases with increasing ligand concentration and that saturation is apparently approached at the highest antigen concentration. A binding constant of about 6.2×10^8 liters/mole (L/M) can be calculated from the free concentration of labeled antigen at which half saturation is attained. This is based on the relation: $r/(n - r) = Kc$ (11), in which r is the number of molecules of ligand bound per unit number of cells, n is the maximum number of molecules of

ligand which that number of cells may bind, K is the binding constant, and c is the free ligand concentration. We have assumed that saturation was achieved at the highest ligand concentration utilized. It is, in fact, difficult to determine n with accuracy because, at higher ligand concentration, there is an appreciable nonspecific binding (see next paragraph).

The specificity of binding is shown in Fig. 2, where cells from immune and nonimmune guinea pigs are compared in their ability to bind $DNP_{16}GPA$-^{125}I.

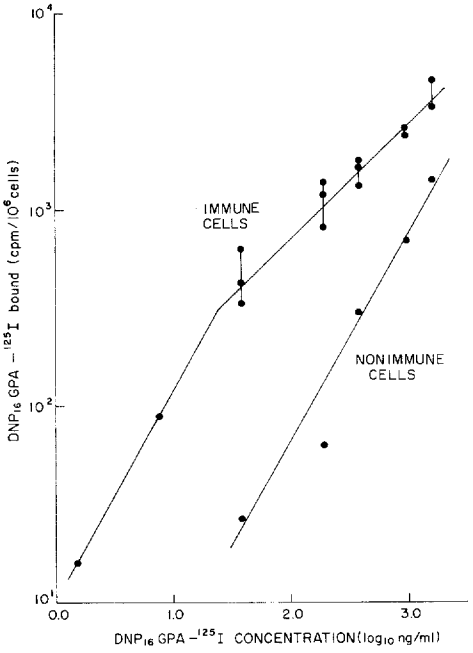

FIG. 2. Specificity of binding of $DNP_{16}GPA$-^{125}I to lymphocytes (see Fig. 1). Cells were obtained from guinea pigs immunized 30 days previously with 50 μg of $DNP_{16}GPA$ in complete Freund's adjuvant (CFA) (immune cells) or CFA alone (nonimmune cells).

It may be seen that at low concentrations of antigen the binding is quite specific but at higher concentrations of $DNP_{16}GPA$-^{125}I the nonimmune cells adsorb significant amounts of ligand. For example, at 40 ng/ml the ratio of binding by immune compared to nonimmune cells is about 20 to 1, whereas at 400 ng/ml the ratio is about 5 to 1. All of the subsequent studies were performed at 200 ng/ml, a concentration at which slightly more than half saturation is attained and at which nonspecific binding is approximately 10% of specific binding.

Estimates can be made of the number of receptors per ABC on the basis of the number of ABC enumerated in a population, the amount of antigen bound by that population, and the number of bonds between antigen and receptor. Table I presents data calculated from several experiments. The geometric

mean of the number of molecules of antigen bound per ABC is 21,000. Assuming that the number of bonds between the cell surface and an individual DNP-GPA molecule is two (see below) and that the receptors are 60–80% saturated, then the number of receptors per cell would be 50,000–70,000. This number is reasonably close to the estimates of Byrt and Ada (12) concerning the binding of flagellin to mouse ABC (17,000–40,000 molecules per ABC) and also agrees with the estimates of Rabellino et al. (13) on the number of immunoglobulin molecules per bone marrow–derived lymphocyte (51,000–143,800). Nonetheless, several approximations in the calculations lead to some uncertainty in

TABLE I

*Number of Molecules of $DNP_{16}GPA$-^{125}I Bound by Antigen-Binding Cells**

Time after immunization	ABC‡ per 10^2 lymphocytes	Molecules DNP_{16}-GPA-^{125}I bound per ABC§
days		
12	1.5	18,000
21	3.3	34,000
21	3.1	28,000
30	1.6	11,000
30	1.2	11,000
30	3.7	13,000
30	4.8	68,000
		21,000 (16,300–27,200)‖

* Cells suspended in 0.2 ml medium containing 200–250 ng of $DNP_{16}GPA$-^{125}I/ml, under conditions described in Materials and Methods.

‡ Lymphoid cells which, by radioautography, have ≥ 3 grains after 1–5 days' exposure time.

§ Concentration of $DNP_{16}GPA$-^{125}I (200–250 ng/ml) should bind 60–80% of receptors (See Fig. 1).

‖ Geometric mean and range encompassed by ± 1 standard error.

the absolute number. Thus, the enumeration of ABC is based on an arbitrary criterion that only cells with more than a certain number of grains are antigen-binding and therefore some ABC may be missed. Secondly, the difficulties in reaching saturation will introduce an error, and thirdly, the number of bonds per molecule is not precisely known. Nonetheless, this estimate appears to be of the proper order of magnitude.

Association and Dissociation Rates of Antigen-Receptor Complexes.—The interaction of antigen and the membrane receptor of specific immunocompetent cells should have the characteristics of a thermodynamically driven process in that it should be time and temperature dependent and be reversible. The time dependency of the association reaction was measured by incubation of lymph node cells in the presence of 0.2 ml of radiolabeled antigen (250 ng of DNP_{16}-GPA-^{125}I/ml) for various periods of time. Further association was stopped by

the addition of a 20-fold excess of unlabeled antigen. If the unlabeled antigen was added to the cell suspension immediately before the addition of the labeled antigen, approximately 95% of the labeled antigen was blocked from binding. Therefore, the unlabeled antigen was an effective means of blocking the association of labeled antigen. Fig. 3 *a* demonstrates the association of $DNP_{16}GPA$-^{125}I to lymph node cells from a guinea pig immunized 18 days before sacrifice

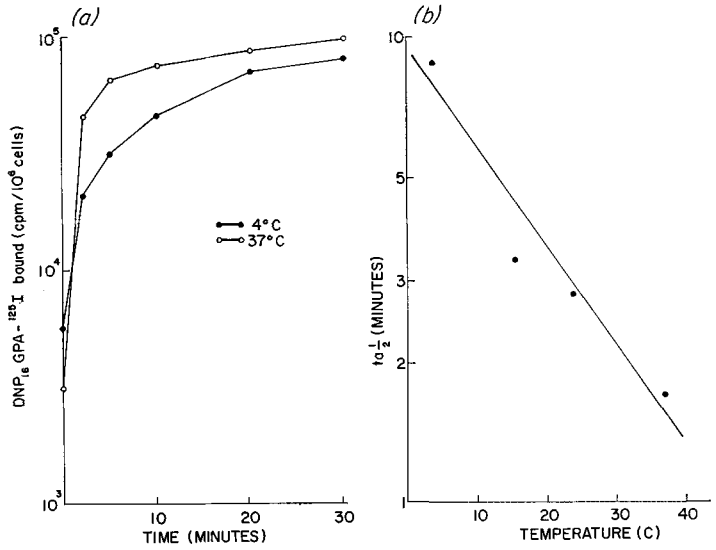

FIG. 3. Association of antigen with cell receptors. Fig. 3 *a*, effect of time and temperature on association rates of antigen-receptor complexes. Lymph node cells from a guinea pig immunized 18 days previously with $DNP_{16}GPA$ in CFA were incubated with 250 ng/ml of $DNP_{16}GPA$-^{125}I under conditions described in Materials and Methods. At various times after incubation either at 4°C or at 37°C, 20-fold excess of unlabeled antigen was added to inhibit further association of labeled antigen. Unbound antigen was removed and the amount of $DNP_{16}GPA$-^{125}I adsorbed to cell receptors was determined. Fig. 3 *b*, effect of temperature on association rates of antigen-receptor complexes. Lymph node cells from a guinea pig immunized 29 days previously were treated as above at several temperatures.

with 50 μg $DNP_{16}GPA$ in complete adjuvant. The association rate was measured at both 4° and 37°C. Each association curve is marked by a rapid initial association phase followed by a slower reaction. The temperature dependence is clear: the time for 50% maximal association at 37°C is 2–3 min, whereas, at 4°C, a comparable degree of association is reached at 11–12 min. A more extensive demonstration of the temperature dependence is provided in Fig. 3 *b* where the time for half-maximal binding is plotted against the temperature. In this experiment, the cells were from guinea pigs immunized 29 days previously. The association rate was measured at four different temperatures. The rate of association is directly related to the temperature.

The dissociation of antigen from membrane-associated receptor was measured as follows: lymph node cells from immunized animals were mixed with a concentration of $DNP_{16}GPA$-^{125}I designed to give approximately half saturation of the receptors (200 ng/ml) and incubated for 1 hr at 4°C. The unbound antigen was removed as usual by centrifugation through fetal calf serum. The washed cell pellet was then resuspended in fresh medium containing 10% fetal calf serum and 1 μg/ml of unlabeled $DNP_{16}GPA$. The cells were incubated at 4°C for various times and then washed one time through fetal calf serum. The amount of labeled antigen which remained associated with the cell pellet was measured in a gamma counter and the degree of dissociation was calculated.

When the cell pellets were incubated for similar periods of time in the absence of unlabeled antigen, no significant dissociation of antigen could be measured. This presumably indicates that any antigen which dissociates from the receptor during this period immediately reassociates with the same or neighboring receptors. Typically, in the presence of excess unlabeled antigen the amount released from the cell receptors increases with time. However, it is clear that the dissociation rate is extremely slow, so that only 16% of the antigen was released in 16 hr from cells of a guinea pig immunized 35 days previously.

It is shown in the accompanying paper (14) that the receptors on a population of ABC obtained shortly after immunization are of much lower average avidity for the antigen than are the receptors of a cell population obtained long after immunization. This change in avidity of the cell receptors parallels the change in affinity of the serum antibody and thus is related to the phenomenon of immunological maturation (15). It would be expected, therefore, that such avidity changes could be measured directly by changes in the association and/or dissociation rates of antigen binding. To test this, lymph node suspensions from guinea pigs immunized 13 days before sacrifice were compared to cells from animals immunized 90 days previously. The association and dissociation rates of these two cell populations are shown in Fig. 4. It is seen that cells from the animals immunized 13 days previously associate relatively slowly with the ligand and dissociate rapidly; the cells from the animals immunized 90 days previously associate more rapidly with $DNP_{16}GPA$-^{125}I and release only insignificant amounts within the limits of observation. Therefore, the receptors of cells from the 90 day animal have higher avidity for $DNP_{16}GPA$-^{125}I than do the cells from the 13 day animal. Numerical values for the rate constants can be estimated using the following formulae (16).

Association rate constant (k_a):

$$k_a = \frac{\ln 2}{t_a 1/2} \cdot \frac{s}{Ag_o}$$

where, t_a ½ is the time in seconds for half-maximal association with antigen, s is the degree of saturation of receptors, and Ag_o is the initial free antigen concentration.

Dissociation rate constant (k_d):

$$k_d = \frac{\ln 2}{t_d 1/2}$$

where, t_d ½ is the time in seconds for half the Ag-receptor complexes to dissociate.

From these values the binding constant can be calculated, as $K = k_a/k_d$. Table II summarizes the rate and binding constants for cells from animals

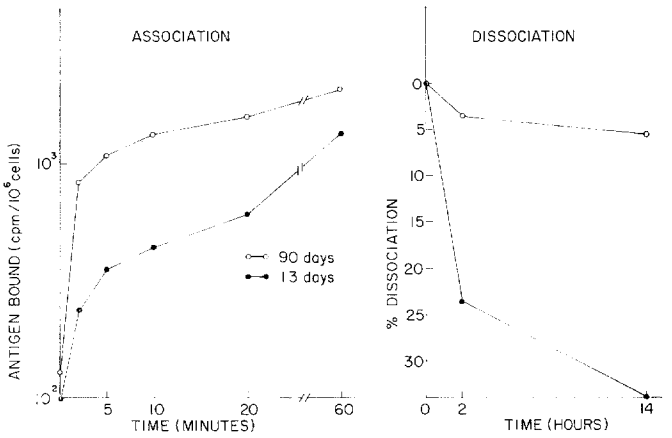

FIG. 4. Effect of time after immunization on the association and dissociation rates of antigen-receptor complexes. Lymph node cells from guinea pigs immunized 13 days and 90 days previously with $DNP_{16}GPA$ in CFA were used. The association rate of the $DNP_{16}GPA$-^{125}I receptor complex was determined (see Fig. 3). Dissociation was measured by incubating $DNP_{16}GPA$-^{125}I cell complexes in unlabeled $DNP_{16}GPA$ (1 μg/ml) at 4°C. At 2 and 14 hr the cells were washed once by centrifugation through fetal calf serum and the amount of antigen associated with the cell pellet was measured. The per cent dissociation of antigen-receptor complexes was then calculated.

immunized 13 and 90 days previously as well as for cells from individual animals immunized 24 and 26 days earlier. The dissociation rate for the "90-day animals" could not be measured by these methods, but an upper limit was obtained. It is clear that with increasing time after immunization, the association rate constant rises and the dissociation rate constant falls. The binding constant (K) of the cells from 90-day animals is more than 100 times that of the cells from 13-day animals. The binding constants measured here by the k_a/k_d ratios are considerably greater than the binding constant as estimated by the reciprocal of the concentration of antigen which binds 50% of the receptors. Thus our estimate for K of receptors of cells of an animal immunized 24 days earlier was 2.7×10^8 L/M by the equilibrium binding method and 2.4×10^{11} L/M by calculation from rate constants. The reason for this large

discrepancy is far from clear but a consideration of some possible causes will be presented in the Discussion section.

Evidence for Binding of Individual Polyvalent Antigen Molecules by More Than One Bond.—The binding of multivalent antigen to cells bearing multiple identical receptors may involve the formation of more than one bond between a single antigen molecule and a single cell. The consequences of such multiple binding are potentially immense since receptors which have a K of 10^5 L/M for monovalent ligands will ideally bind a divalent ligand with a K of 10^{10} L/M if each molecule is bound by two receptors. The contribution of cooperative binding to the K obtained using multivalent ligands would best be determined by directly measuring the K of binding of monovalent antigen

TABLE II

Effect of Time after Immunization on Rate and Binding Constants

Time after immunization	k_a (10^5 M^{-1} sec^{-1})	k_d (10^{-7} sec^{-1})	K^* (L/M)	$K\ddagger$ (L/M)
days				
13	1.4	19.0	7.6×10^{10}	
24	2.2	9.2	2.4×10^{11}	2.7×10^8
26	2.0	1.0	2.0×10^{12}	
35				6.2×10^8
90	8.1	<1.0	$>8.1 \times 10^{12}$	

* $K = k_a/k_d$.

‡ $K = 1/C$ where C is free ligand concentration when half of receptors are bound.

to cells. Our attempts to measure direct binding of mono-DNP compounds to the receptors of cells from immunized animals were unsuccessful, however. We have used two compounds in these experiments, ε-DNP–L-lysine-^{3}H (25 Ci/mM) and DNP-caproyl-β-alanyltyrosine-^{125}I (prepared by Dr. John Inman of the National Institutes of Health). No significant binding of either of these compounds could be measured after the washing procedures were performed, suggesting that the affinity of these compounds for ABC was considerably lower than that of polyvalent ligands. Indirect evidence for binding of monovalent ligands to cell receptors can, however, be easily obtained. When sufficient free ε-DNP–L-lysine is added to cell suspensions before and during exposure to $DNP_{16}GPA$-^{125}I, inhibition of binding of the multivalent ligand occurs (Fig. 5). This effect is undoubtedly due to competition of the two DNP compounds for receptor sites. A measure of the relative avidity of the receptors for a series of DNP compounds can be obtained by determining the molar concentration of each of the DNP compounds needed to inhibit 50% of DNP_{16}-GPA-^{125}I from binding to the cells and comparing these 50% inhibitory concentrations (I_{50}).

Using the inhibitory assay to determine relative avidity, the effect of multi-

valency on avidity was measured. A series of DNP conjugates of BSA was prepared. By using a single protein carrier and varying the number of hapten molecules associated with it, it was hoped that variation in structure and size of the molecules could be minimized so that only DNP density would vary. Fig. 6 summarizes the effect of varying DNP substitution on the relative inhibitory capacity of DNP-BSA on $DNP_{16}GPA$-^{125}I binding. By increasing the substitution from 4 to 19, approximately a 100-fold diminution in the I_{50} value, as measured per mole of BSA, was achieved. Any increase beyond 19

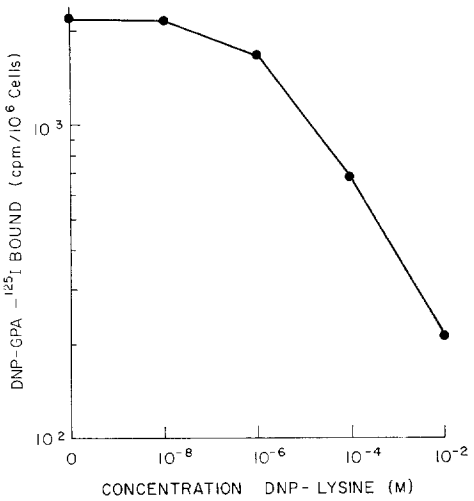

FIG. 5. Inhibition by DNP-lysine of $DNP_{16}GPA$-^{125}I binding to cell receptors. Lymph node cells from a guinea pig immunized 18 days previously with $DNP_{16}GPA$ in CFA were obtained mixed with $DNP_{16}GPA$-^{125}I (250 ng/ml) and various concentrations of ε-DNP-L-binding of $DNP_{16}GPA$-^{125}I to cell receptors was determined as described in Materials and Methods.

groups per molecule did not further increase the avidity (diminish the I_{50} value). One interpretation of this result is that the increase in avidity with increased DNP density reflects the frequency of DNP-BSA molecules possessing DNP groups spaced an appropriate distance apart to bridge two anti-DNP receptors. These two receptors could well be the two Fab′ portions of a single receptor molecule, although this is purely conjecture. Indeed, it is quite possible that more than two receptors bind to an individual DNP-BSA molecule, but direct evidence for this is not available.

If multivalent DNP-BSA spans a limited number (two?) of Fab′ receptors because of its relatively small molecular dimensions, then very large multivalent molecules may be able to bridge more receptor molecules and be bound more avidly than DNP-BSA. Therefore, the I_{50} of DNP conjugates of varying molecular weights was measured.

Table III summarizes three experiments in which the relative I_{50} values of $DNP_{16}GPA$ and $DNP_{109}BF$ (mol wt $> 1 \times 10^6$) are compared to that of DNP-lysine. The larger compound is a more effective inhibitor of $DNP_{16}GPA$-^{125}I binding than is $DNP_{16}GPA$ itself; the increased effectiveness of inhibition is about 25-fold, when measured per molecule, assuming the molecular weight of the DNP-BF to be 1×10^6. $DNP_{16}GPA$ and $DNP_{109}BF$ are essentially equiva-

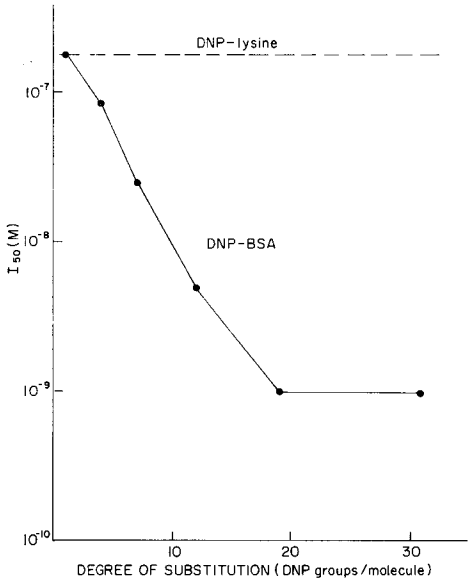

FIG. 6. Effect of the degree of DNP substitution on the relative binding capacity of DNP-BSA to cell receptors. Lymph node cells from a guinea pig immunized 58 days previously with $DNP_{16}GPA$ in CFA were used. The I_{50} of DNP-BSA preparation of different degrees of substitution was determined as described in the Results section.

lent when considered per DNP group and both are superior to ε-DNP-L-lysine. The increased binding avidity, per molecule, shown by $DNP_{109}BF$, compared to $DNP_{16}GPA$, suggests that some of the former molecules may participate in more interactions than do $DNP_{16}GPA$ molecules. This interpretation is clearly preliminary and requires the use of more precisely constructed ligands with known haptenic spacing to be confirmed.

Binding of more than a single DNP group per molecule to receptors of a cell should also be reflected both in the number of molecules bound and in the dissociation rates. DNP_4BSA-^{125}I, $DNP_{16}GPA$-^{125}I, and DNP_9KLH-^{125}I were compared in these parameters. Table IV summarizes the results. At the same molar concentrations, 10 times more $DNP_{16}GPA$ than DNP_4BSA was bound, and the $DNP_{16}GPA$ was held more tightly as shown by the slower dissociation rate. Due to the molecular weight heterogeneity of DNP_9KLH, no estimate

of the number of molecules bound was attempted. However, the dissociation rate of the DNP-KLH conjugate is markedly slower than that of $DNP_{16}GPA$

TABLE III

*Relative Capacities to Inhibit $DNP_{16}GPA$-^{125}I Binding of DNP Compounds of Varying Molecular Weights**

Experiment	Days after immunization	Inhibitor	Molecular weight	I_{50}	Relative inhibitory capacity	
					Per molecule	Per DNP group
				ng/ml		
1	22	DNP-lysine	313	720	1	1
2	55			36	1	1
3	56			36	1	1
1	22	$DNP_{16}GPA$	74,000	280	590	37
2	55			43	200	12
3	56			66	130	8
				Geo. mean	250	15
1	22	$DNP_{109}BF$	$>1 \times 10^6$‡	420	5,700	19
2	55			8	14,900	50
3	56			43	2,800	9
				Geo. mean	6,200	20

* Lymph node cells from animals immunized with $DNP_{16}GPA$ were exposed to $DNP_{16}GPA$-^{125}I (200 ng/ml) and varying concentrations of unlabeled inhibitors under conditions described in Materials and Methods. The concentration of inhibitor needed to block 50% of $DNP_{16}GPA$-^{125}I from binding (I_{50}) was calculated.

‡ In the calculation of I_{50} per molecule of DNP-BF, a molecular weight of 1×10^6 was assumed.

TABLE IV

Effect of Multivalent Binding to Cell Receptors on the Amount of Antigen Bound and on the Dissociation Rate

Ligand	Molecules antigen bound/10^6 cells	Time for 20% dissociation
		hr
DNP_4BSA-^{125}I*	1.3×10^8	2.9
$DNP_{16}GPA$-^{125}I*	12.8×10^8	9.1
DNP_9KLH-^{125}I	—‡	38.0

* 1×10^{-9} M.

‡ Not calculated because of uncertainty in molecular weight.

or DNP_4BSA. It is not clear, however, whether the slower dissociation rate exhibited by DNP_9KLH simply represents divalent binding (via two Fab′ fragments) by the highest avidity subpopulation of receptors, rather than additional cooperative interaction by separate immunoglobulin molecules.

DISCUSSION

The binding of antigen to antigen-specific cellular receptor is a key event in the initiation and regulation of the immune response. It is this antigen-cell interaction which imparts to the immune system its main attribute, specificity. Moreover, the various outcomes which may follow cell-antigen interaction (proliferation, differentiation, inactivation) have been postulated to depend, in large part, on the manner of the presentation of antigen to cells (17, 18). It seems quite clear that progress in the understanding of cellular activation by antigen requires a precise delineation of the nature of the interaction between antigens and receptors.

In this study, we have presented a direct approach to the analysis of the binding of an ^{125}I-labeled antigen, $DNP_{16}GPA$-^{125}I, to receptors on lymphocytes of immunized animals. This multivalent antigen binds to cell surfaces in a manner characteristic of a reversible, thermodynamically driven reaction. The ligand appears to depend upon a synthesized product of the lymphocyte and not to be due to passive coating of the cells by a cytophilic antibody (5). Although it seems evident that lymphocytes of both the bone marrow–derived (B) and thymus-derived (T) lines undergo surface interactions with antigen (19), the cells detected by the antigen-binding method we have utilized appear to be members of the B line, on the basis of their possession of large amounts of surface immunoglobulin. It seems that specific T cells bear many fewer surface receptor molecules (13).

A good correlation exists between the amount of antigen bound by an immune cell population and the number of ABC within that population (5). Indeed, by dividing the amount of antigen bound by the number of ABC, an estimate of 50,000–70,000 receptors per ABC is obtained. This is reasonably similar to the estimates made by Rabellino et al. (13) as to the number of immunoglobulin molecules per bone marrow–derived lymphocyte in mice (51,000–143,000).

The binding parameters studied in this paper include association rates, dissociation rates, equilibrium constants, and the question of bivalent or multivalent binding. Certain of these factors bear comment. Association rates were temperature dependent and increased with time after immunization. Dissociation rates were exceedingly slow; indeed, for cells obtained from animals immunized 90 days earlier, these rates were essentially unmeasurable. As discussed below, it appears that the discrepancy in K from equilibrium binding data and from rate constants may, in part, be due to the slow dissociation rate. Moreover, the dissociation rate suggests that cells with high affinity receptors might retain surface antigen for a prolonged period of time. A series of possible roles for such retained antigen could be entertained and consequently a search for antigen associated with lymphocyte surfaces is now being made.

Two important points follow from the rapid association rate and slow dissociation rate. The most obvious, of course, is that the equilibrium constant is

very high; moreover, this constant increases with time after immunization in a manner similar to that of the affinity of serum antibody for univalent ligand and its avidity for multivalent ligands. This change in receptor avidity, indeed, provides the most convincing evidence that the time-dependent affinity changes of antibody reflect selection of precursors of antibody-secreting cells bearing receptors of given binding characteristics.

Before further considering the biologic implication of these studies, the problem of the marked difference in K value obtained by the two methods requires discussion. To begin, it is clear that the approach of treating multivalent antigen and cell surfaces as ideal gases is an enormous simplification and one in which uncontrolled variables exist which may easily account for these differences. Nonetheless, several possible causes should be explicitly considered. Firstly, the K determined by equilibrium binding is obtained on the basis of the free ligand concentration required for half saturation and thus reflects all of the cell receptors. The association and dissociation rates are calculated from the behavior of $\sim$60% of the receptors, these being the most avid. Therefore, because of heterogeneity in the receptor population the K value obtained by the k_a/k_d would certainly be somewhat greater than the K from equilibrium binding. Furthermore, if heterogeneity in the antigen exists, this would lead to the binding of the most avid subfraction and consequently to a slow dissociation rate, again yielding a higher K value by the k_a/k_d method. Finally, after the binding of antigen to the cell surface, additional slow reactions may occur which stabilize the complex leading to a slow dissociation rate. Whether the cumulative effects of these three mechanisms can explain the discrepancy is not certain. Nevertheless, by considering K values obtained by either method, an increase in avidity of receptors for antigen occurs in the course of the immune response.

The superiority of multivalent $DNP_{16}GPA$ over univalent ε-DNP-L-lysine in both direct binding and in competitive inhibition of binding strongly suggests that more than one bond is formed between the $DNP_{16}GPA$-^{125}I and the ABC. Although one cannot definitively determine how many bonds are formed initially, an approach may be made from two directions. First the measured K of the antigen-cell interaction, whether considered by the k_a/k_d method or the equilibrium binding method, is no more than the square of the K for the binding of antibody by univalent ligand (10^{+5}–10^{+8}). Theoretically, the total standard free energy (ΔF^0) of binding between two particles is the sum of the ΔF^0 of each bond. Since $\Delta F^0 = -RT \ln K$, where R is the gas constant and T is the absolute temperature, the equilibrium constant should be that of each bond raised to a power equivalent to the number of bonds. Although obviously a set of nonideal forces may be involved, the difference in K exhibited by ε-DNP-L-lysine and $DNP_{16}GPA$-^{125}I suggests that two bonds may be initially involved.

Alternatively, if the receptor molecules are uniformly spaced on the cell surface, one can estimate the distance between them and compare it to the

molecular size of the ligand. Assuming an average diameter of 7 μ/ABC, a spherical configuration and no folding of the membrane, the surface should be 154 μ^2. This is obviously an underestimate as considerable membrane irregularity exists. Nonetheless, if we take this low estimate and further assume a value of 50,000–70,000 receptors/cell, we can calculate the average distance between receptors. First, assuming that the receptors exist as divalent molecules, about 30,000 points/154 μ^2 would exist; each point would be in the center of a circle with an area of 0.0051 μ^2. The radius of this circle would be 0.040 μ and thus each point would be separated by 0.080 μ or 800 A. The longest axis of serum albumin is approximately 145 A (20) and the maximum length of an Fab fragment is 65 A (21) suggesting that, even neglecting the folding of the membrane, DNP-GPA should not span two surface immunoglobulin molecules, if they are evenly spaced. Again, therefore, a maximum of two bonds between an ABC and a single $DNP_{16}GPA$ molecule would be expected. One major objection to this latter approach is the observation by immunofluorescent techniques that immunoglobulin on the surface of cells exists in spots or patches (4, 13). This would potentially allow the formation of many more bonds. However, it is not clear whether immunoglobulin exists as spots on the cell surface normally or whether spot formation occurs after the binding of the fluorescent anti-immunoglobulin reagents.

In any event, the increments in binding energy observed result in high equilibrium constants for the interaction of multivalent antigen with ABC. Since many naturally occurring antigens of pathogenetic importance are large molecules with repeating determinants (viruses, bacterial flagella and polysaccharides, cell surface neo-antigens), the energetic advantages of multivalent binding may have considerable biologic importance in that ABC with receptors of relatively low affinity for an individual determinant could, nevertheless, bind the polyvalent antigen exceedingly efficiently. Similar considerations concerning the binding of multivalent antigens to divalent serum antibody have been previously developed (22, 23).

Finally, in the understanding of antigen-mediated activation of precursors of antibody-forming cells, the role of the specific thymus-derived cell (T cell) is a major problem. Certain molecules exist which, in the mouse at least, do not require thymus-derived cells for activation of precursors of antibody-forming cells. Feldmann and Basten have demonstrated that DNP conjugates of polymerized flagellin can activate precursors of antibody-secreting cells in the relative absence of T cells, whereas DNP-BSA requires the presence of specific T cells for measurable activation of precursors (24). The analysis of the relative capacity of cells to bind DNP conjugates of "thymus-dependent" carriers and of "thymus-independent" carriers may therefore allow a determination to be made if the differences between such materials lies in the effective energy of interaction between antigen and precursor cell surface and if a role for the thymus-dependent cell is to increase this binding energy by presenting the antigen

in a more favorable array. Such studies appear now to be quite feasible to porform and preliminary experiments on this point are in progress.

SUMMARY

The interaction of antigen with specific, cell-associated receptors was measured in thermodynamic terms. The binding of ^{125}I-labeled 2,4-dinitrophenyl guinea pig albumin ($DNP_{16}GPA$-^{125}I) to lymphocytes from guinea pigs immunized to $DNP_{16}GPA$ is a temperature-dependent, reversible process. Measurement of association and dissociation rates of antigen-receptor complexes permits calculation of antigen-cell binding constants. These may also be calculated by equilibrium-binding techniques. Although differences in the constants calculated in these two ways exist, a clear increase in avidity of cell receptor for antigen occurs in the course of the immune response. This change in receptor avidity provides evidence that the time-dependent change in affinity of serum antibody (maturation) indeed has a cellular basis.

The magnitude of the equilibrium constant is, in part, due to binding of more than one DNP group per molecule of antigen. Thus, multivalent ligands bind more effectively to cell receptors than univalent or paucivalent ligands when measured by the number of antigen molecules bound, the dissociation rate of antigen-receptor complexes, and in the relative capacity to inhibit a standard multivalent ligand ($DNP_{16}GPA$-^{125}I) from binding.

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REFERENCES

1. Naor, D., and D. Sulitzeanu. 1967. Binding of radioiodinated bovine serum albumin to mouse spleen cells. *Nature (London).* **214:**687.
2. Warner, N. L., P. Byrt, and G. L. Ada. 1970. Blocking of the lymphocyte antigen receptor site with anti-immunoglobulin sera *in vitro*. *Nature (London).* **226:**942.
3. Humphrey, J. H., and H. U. Keller. 1970. Some evidence for specific interaction between immunologically competent cells and antigens. *In* Developmental Aspects of Antibody Formation and Structure. J. Sterzl and I. Riha, editors. Academia Publishing House of the Czechoslovak Academy of Sciences, Prague. **2:**485.
4. Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptors on dinitrophenylated guinea pig albumin–^{125}I-binding lymphocytes of normal guinea pigs. *J. Exp. Med.* **134:**495.
5. Davie, J. M., A. S. Rosenthal, and W. E. Paul. 1971. Receptors on immunocompetent cells. III. Specificity and nature of receptors on dinitrophenylated guinea pig albumin–^{125}I-binding cells of immunized guinea pigs. *J. Exp. Med.* **134:**517.
6. Ada, G. L. and P. Byrt. 1969. Specific inactivation of antigen-reactive cells with ^{125}I-labeled antigen. *Nature (London).* **222:**1291.
7. Wigzell, H., and O. Mäkelä. 1970. Separation of normal and immune lymphoid

cells by antigen-coated columns. Antigen-binding characteristics of membrane antibodies as analyzed by hapten-protein antigens. *J. Exp. Med.* **132:**110.

8. Schwert, G. W. 1957. Recovery of native bovine serum albumin after precipitation with trichloroacetic acid and solution in organic solvents. *J. Amer. Chem. Soc.* **79:**139.
9. Benacerraf, B., and B. B. Levine. 1962. Immunological specificity of delayed and immediate hypersensitivity reactions. *J. Exp. Med.* **115:**1023.
10. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89:** 114.
11. Karush, F. 1962. Immunologic specificity and molecular structure. *Advan. Immunol.* **2:**1.
12. Byrt, P., andG. L. Ada. 1969. An *in vitro* reaction between labeled flagellin or haemocyanin and lymphocyte-like cells from normal animals. *Immunology.* **17:**503.
13. Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1971. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* **133:**156.
14. Davie, J. M., and W. E. Paul. 1972. Receptors on immunocompetent cells. V. Cellular correlates of the "maturation" of the immune response. *J. Exp. Med.* **135:**660.
15. Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry.* **3:**996.
16. Talmage, D. W. 1960. The kinetics of the reaction between antibody and bovine serum albumin using the Farr method. *J. Infec. Dis.* **107:**115.
17. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Advan. Immunol.* **10:**1.
18. Mitchison, N. A. 1967. Antigen recognition responsible for the induction in vitro of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32:**431.
19. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus-derived (T) and non-thymus derived (B) lymphocytes by ^{125}I-labeled antigen. *Nature (New Biol.) (London).* **231:**104.
20. Phelps, R. A., and F. W. Putnam. 1960. Chemical composition and molecular parameters of purified plasma proteins. *In* The Plasma Proteins. F. W. Putnam, editor. Academic Press, Inc., New York. **1:**143.
21. Green, N. M. 1969. Electron microscopy of the immunoglobulins. *Advan. Immunol.* **11:**1.
22. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1966. Studies on the effect of the carrier molecule on antihapten antibody synthesis. II. Carrier specificity of anti-2,4-dinitrophenyl poly-L-lysine antibodies. *J. Exp. Med.* **123:**689.
23. Hornick, C. L., and F. Karush. 1969. The interaction of hapten-coupled bacteriophage $\varphi \times 174$ with antihapten antibody. *Israel J. Med. Sci.* **5:**1963.
24. Feldmann, M., and A. Basten. 1971. The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. *J. Exp. Med.* **134:**103.