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Antigen-Specific Mouse Lymphocyte Stimulation by DNP-Conjugated T-Independent Antigens Studied by Photobleaching Recovery

James S. Peacock and B. George Barisas

Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

Fluorescence photobleaching recovery techniques have allowed us to measure the lateral mobility of T-independent antigens bound to antigen-specific mouse B cells. The in vitro immunogenicity or tolerogenicity of antigens we have examined, DNP-polymerized flagellin (DNP-POL), and DNP-linear dextran (DNP-DEX), depend upon the antigen dose and epitope density. These factors also determine the mobility of antigen bound to B cell surfaces. For DNP-POL bound to DNP-specific cells, the observed diffusion constants D decrease monotonically with increasing antigen dose and epitope density. Values of D range from 10.4×10^{-11} cm^2 sec^{-1} for $DNP_{0.4}$-POL at 0.15 μg/ml to 0.8×10^{-11} cm^2 sec^{-1} for $DNP_{3.5}$-POL at 30 μg/ml. For receptor-bound DNP-DEX, D depends strongly on antigen epitope density but not observably on antigen concentration. For epitope densities of 1.2 or less, D is close to the value of 21×10^{-11} cm^2 sec^{-1} observed for single sIg receptors. By an epitope density of 4.8, D has fallen to 2.1×10^{-11} cm^2 sec^{-1}. Peak immunogenicities for DNP-POL and DNP-DEX are observed when antigen- receptor aggregates have mobilities 14-fold and 3-fold lower, respectively, than a single sIg molecule.

Key words: DNP, T-independent, flagellin, fluorescence photobleaching recovery, stimulation, photobleaching

Abbreviations used: PBS, phosphate-buffered saline (0.14 M sodium chloride, 0.01 M sodium phosphate, pH 7.4); MEM, modified Eagle's minimum essential medium; POL, polymerized S adelaide flagellin; FITC, fluorescein isothiocyonate; SRBC, sheep red blood cells; MON, S adelaide flagellin monomer; HBSS, Hank's balanced salt solution; TRITC, tetramethylrhodamine isothiocyanate; AFC, antibody-forming cells; sIg, surface immunoglobulin; DEX, linear dextran of mol wt 2×10^6 daltons; FPR, fluorescence photobleaching recovery; TI, thymus-independent.

James S. Peacock and B. George Barisas are now at the Departments of Chemistry and Microbiology, Colorado State University, Fort Collins, CO 80523.

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Direct interactions between T-independent (TI) antigens and immunoglobulin molecules on B cell membranes can either stimulate or tolerise the responding cells. Consequently, antigen–receptor interactions and subsequent dynamic events on the lymphocyte membrane have been the subject of great interest. TI antigens are, in general, large polymeric molecules with repeating determinants. The frequency of these determinants along the polymer (epitope density) and antigen dose were shown to be extremely important in determining the type of physiologic effects produced by a given antigen [1]. How these parameters regulate the antigen–receptor interactions and membrane responses that determine whether immunogenic or tolerogenic signals are delivered to the cell remains unclear. However, one measurable quantity that directly reflects antigen–receptor interaction is the lateral mobility of membrane bound receptor–antigen complexes. That antigen binding greatly affects the lateral dynamics of membrane components is clearly demonstrated by the receptor aggregation observed visually during lymphocyte capping [2]. On the other hand, an abundance of data indicates that this process is neither necessary nor sufficient for cellular activation [3, 4]. The receptor cross-linking involved in T-independent antigen stimulation of specific B cells must be a more subtle process. The technique of fluorescence photobleaching recovery (FPR) has been developed in recent years [5] to measure directly the lateral mobility and mobile fraction of specific molecules on single, viable cells. We describe here measurements by FPR of how the lateral mobilities of two T-independent antigens bound to DNP-specific mouse B cells vary between immunogenic and tolerogenic situations and with antigen dose and epitope density. Both materials are known to induce dose- and epitope density-dependent TI responses [1, 6]. One of the antigens, DNP-polymerized flagellin (DNP-POL), is a rigid, rod-like molecule, and its binding to the cell surface can be treated thermodynamically by straightforward extension [7] of existing theories [8]. The other antigen, DNP-linear dextran (DNP-DEX), is extremely flexible and exists in solution as a random coil. This material must lose considerable configurational entropy upon binding. It has been suggested [9] that these configurational entropy effects should cause receptor interactions of DNP-DEX to depend differently on epitope density than those of DNP-POL. We therefore also examined the similarities and differences these two antigens exhibit in their interactions with B cell surfaces when studied by FPR.

METHODS

Antigens

Crystalline bovine serum albumin (BSA) was obtained from Sigma Chemical Company and used without further purification. Polymeric flagellin (POL) was prepared from the flagella of S adelaide strain SP.10718 (ACTC) according to the method of Ada et al [10]. Dextran T2000 of molecular weight 2×10^6 was purchased from Pharmacia Fine Chemicals. BSA was dinitrophenylated as described by Eisen [11]. Monomeric flagellin (MON) and POL were dinitrophenylated as described by Feldmann [12] and conjugated with tetramethylrhodamine isothiocyanate (TRITC) by the method of Rinderknecht [13]. Dextran was dinitrophenylated as described by Axen et al [14], cyanogen bromide treated dextran being reacted with ϵ-DNP-lysine. Epitope densities for both antigens are reported as

DNP per 40,000 molecular weight. Some samples of DNP-conjugated dextran were conjugated with fluorescein isothiocyonate (FITC) according to the method of Fernandez [15].

Tissue Culture

Spleen cells from female Swiss Webster mice aged 2–3 months were cultured with DNP antigens as described by Marbrook [16] in 5% CO_2 atmosphere. After 4 days of culture the cells were harvested and counted for anti-DNP antibody-forming cells by direct hemolytic plaque assay [17].

Enrichment of DNP-Specific Cells

DNP antigen-binding mouse spleen cells were enriched either by a modification [18] of the procedure of Gold [19] involving a DNP-gelatin column or by the method of Haas and Layton [20] involving DNP-gelatin coated Petri dishes.

Labeling of Cells With Fluorescent Probes

Approximately 1×10^6 cells enriched for DNP binding cells were cultured overnight in 1 ml of modified Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum. These cells were then washed and suspended in 1 ml of Hank's balanced salt solution (HBSS) containing 20–50 μg of unconjugated POL or DEX and, in some cases, 0.1% BSA. After 30 min at 0°C, various doses of TRITC-DNP-POL or FITC-DNP-DEX were added and further incubated at 0°C for at least 30 min. The cells were washed three times with HBSS immediately prior to FPR measurements.

FPR Measurements

Details of our FPR system shown in Figure 1 are described elsewhere [21, 22]. A light beam (488 nm for FITC-DNP-DEX and 514 nm for TRITC-DNP-POL) from an argon ion laser is divided into weak and intense components by the pulse generator. The intense beam, which is between 10^3 and 10^4 times as intense as the interrogation beam, is blocked by an electronic shutter. Opening the shutter produces bleaching pulses of 5 msec to 8 sec. Lymphocytes were examined under coverslip on well slides at 37°C using a 50X water immersion objective. The DNP specificity of cells labeled by fluorescent DNP antigens has been confirmed previously [21] by conventional immunofluorescence techniques. We have normally used a 0.5-sec bleach and a 240-sec recovery at 1 sec per point for each measurement. On-line analysis of data was performed by NOVA 3/12 minicomputer to yield the diffusion constant and the mobile fraction of cell-bound fluorescent molecules.

RESULTS

Primary Responses to DNP Antigens In Vitro

In vitro anti-DNP responses to various doses and epitope densities of DNP-POL and FITC-DNP-DEX are shown in Figures 2 and 3, respectively. In experiments not described we have found that FITC conjugation of DNP-DEX increases the epitope density required to produce the same responses as the unconjugated antigen. TRITC conjugation of DNP-POL produced no effect. Responses to these

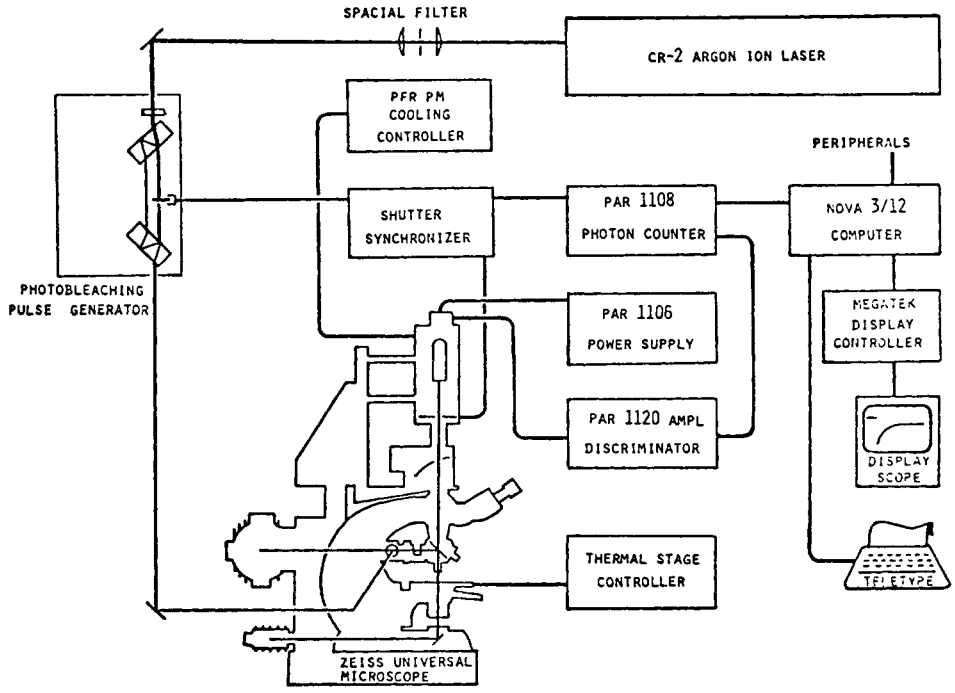

Fig. 1. Block diagram of fluorescence photobleaching recovery system described in text.

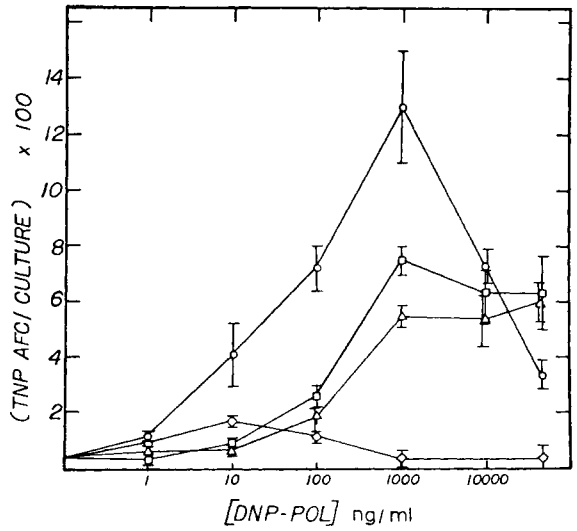

Fig. 2. In vitro primary antibody response of mouse lymphocytes to DNP-POL antigens of various epitope densities. Assay conditions are as described in text. Triangles, squares, circles, and diamonds represent epitope densities of 0.4, 0.8, 2.3, and 3.5 DNP per 40,000 molecular weight, respectively.

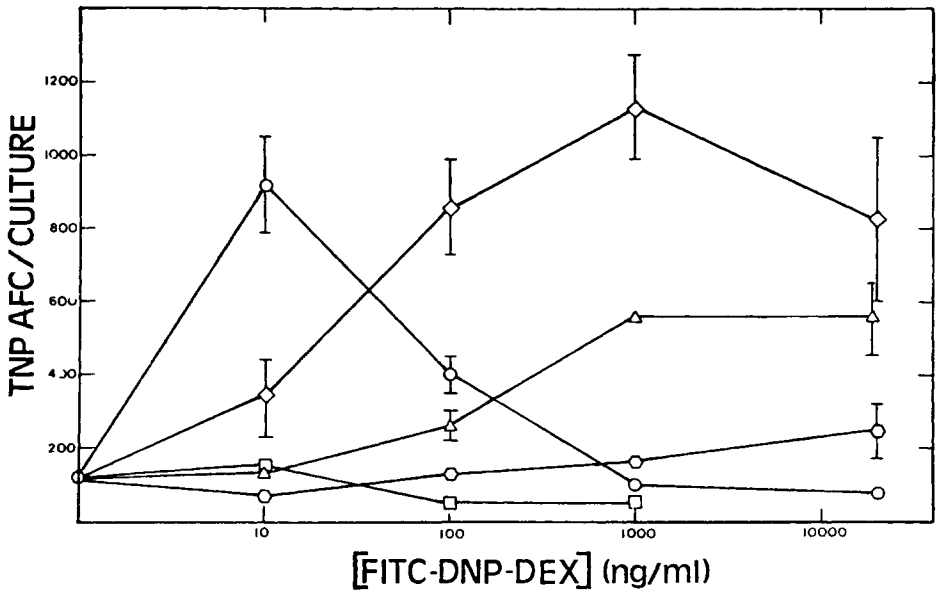

Fig. 3. In vitro primary antibody response of mouse lymphocytes to DNP-dextran antigens of various epitope densities. Assay conditions are as described in text. Hexagons, triangles, diamonds, circles, and squares represent epitope densities of 0.7, 1.2, 3.2, 4.8, and 9.0 DNP per 40,000 molecular weight, respectively.

antigens, as shown by Feldmann [1] and Desaymard and Feldmann [6], depend strongly on the antigen dose and epitope density. Although FITC-DNP-DEX requires higher epitope density than DNP-POL to produce similar effects, both antigens can be divided on the basis of epitope density, into three categories. First, lightly conjugated antigens (0.4 and 0.8 DNP groups per monomer POL and 0.7 and 1.2 DNP groups per 40,000 dalton DEX) produce peak or near peak response at the highest dose tested (30 μg/ml). Second, $DNP_{2\cdot3}$-POL (2.3 DNP groups per monomer) at 1 μg/ml, FITC-$DNP_{3\cdot2}$-DEX (3.2 DNP groups per 40,000 daltons) at 1 μg/ml, and FITC-$DNP_{4\cdot8}$-DEX at 10 ng/ml induce strong responses, but these responses fall sharply at either higher or lower antigen concentrations. Finally, the highest epitope densities tested ($DNP_{3\cdot5}$-POL and FITC-$DNP_{9\cdot0}$-DEX) induce no positive response at any concentration between 10 ng/ml and 30 μg/ml. Lack of immunogenicity of these latter antigens with high epitope density was shown by Feldmann et al [1, 6] to be an active suppression of the target cells and not due to changes in the antigenic structures caused by high degree of hapten conjugation.

Effects of Epitope Density and Antigen Dose on the Lateral Mobility of Receptor-Bound Antigens

Increasing the epitope density of TRITC-DNP-POL and FITC-DNP-DEX reduces the mobility of these antigens bound to DNP receptors on B cell membranes. We [21] have recently shown that the diffusion constant of $DNP_{0\cdot5}$-MON bound to DNP receptors on the mouse B cell (2.2×10^{-10} cm^2 sec^{-1}) equals the

mobility of a single surface immunoglobulin molecule. We can define a mobility reduction index for bound antigen as the diffusion constant of $DNP_{0.5}$-MON or sIg divided by the diffusion constant of bound antigen. Figure 4 shows this index plotted vs antigen concentration for various epitope densities of DNP-POL. The values range from 2 for TRITC-$DNP_{0.4}$-POL at 0.15 μg/ml to 27 for TRITC-$DNP_{3.5}$-POL at 30 μg/ml. The mobility reduction index increases approximately linearly with increasing epitope density and concentration of POL antigens. The mobility reduction index of 14 observed at the optimum immunogenic condition ($DNP_{2.3}$-POL at 1 μg/ml) lies within the values of 13 and 18 that were observed under tolerogenic conditions, $DNP_{3.5}$-POL at 0.1 μg/ml and at 1.0 μg/ml respectively. This demonstrates that, although a significant immunogenic response to POL antigens is observed only when the mobility of receptor-bound antigens decreases manyfold relative to that of sIg, there is no simple relationship between mobilities of *diffusible* antigen–receptor complexes and immunogenicity or tolerogenicity of the antigen. What is clear, however, is that the percentage recovery of fluorophores after photobleaching (Table I) that represents the fraction of antigen-bound receptors mobile on the time scale of the experiment remains at about 50–60% in all immunogenic cases of POL antigens but falls abruptly to 24–32% in those cases where the epitope density–dose combination is tolerogenic.

With FITC-DNP-DEX antigens, substantially different effects of dose and epitope density on the mobility of B cell-bound antigen are observed. In contrast to POL antigens, the index of mobility reduction, presented in Table II, remains constant at about 1 for epitope densities between 0.24 and 1.2 despite the fact that a significant immune response is elicited by the latter antigen. Furthermore, unlike POL antigens, approximately 40% of the total change in mobility reduction index observed between epitope densities 0.24 and 9.0 occurs within a narrow epitope density range of 3.2 and 4.8. The mobility reduction index observed at the optimum immunogenic condition for those antigens (FITC-$DNP_{3.2}$-DEX at 1 μg/ml) is 2 compared with 14 observed for POL antigens.

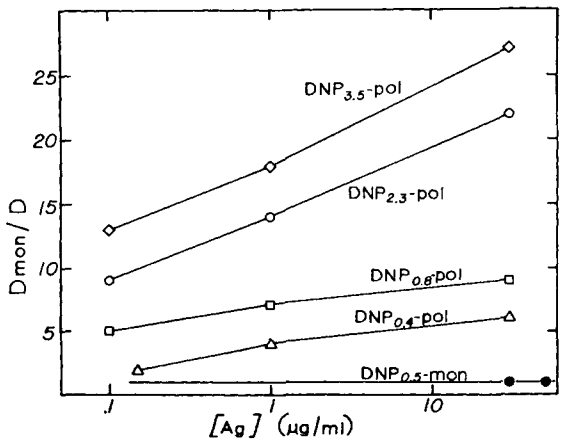

Fig. 4. Diffusion constant of receptor-bound DNP-POL as a function of antigen concentration and epitope density. Data are presented as the ratio of the mobility of a single sIg receptor to that of the bound antigen.

The concentration dependence of antigen-receptor complex mobilities for DEX antigens is not clear. We were unable to test a wide range of concentrations owing to the difficulty in labeling at low concentrations. However, from the available FPR data (Table II) three observations may be made. First, there is no apparent effect of antigen concentration on mobilities of these antigens on B cell surfaces. This is in marked contrast to POL antigens where a 300-fold increase in the concentration results in about a 2½-fold mobility reduction (Fig. 4). Second, unlike POL antigens, there are notable differences between mobilities of diffusible receptor-antigen aggregates in immunogenic and tolerogenic conditions. This is clearly demonstrated in Figure 5b where, at about 30 μg/ml immunogenicity ceases when diffusion constants fall below about 8×10^{-11} cm^2 sec^{-1}. On the other

TABLE I. Fraction of Receptor-Bound TRITC-DNP-MON and TRITC-DNP-POL Mobile in Photobleaching Recovery Experiments

Antigen	Concentration	Percent fluorescence recovery after photobleaching
TRITC-DNP$_{0.5}$-MON	30 μg/ml	56.6 ± 13.6
TRITC-DNP$_{0.5}$-MON	160 μg/ml	52.5 ± 11.3
TRITC-DNP$_{0.4}$-POL	0.15 μg/ml	58.5 ± 14.3
TRITC-DNP$_{0.4}$-POL	1 μg/ml	56.6 ± 9.3
TRITC-DNP$_{0.4}$-POL	30 μg/ml	56.3 ± 10.1
TRITC-DNP$_{0.8}$-POL	0.1 μg/ml	61.5 ± 12.5
TRITC-DNP$_{0.8}$-POL	1 μg/ml	54.1 ± 11.6
TRITC-DNP$_{0.8}$-POL	30 μg/ml	58.3 ± 7.1
TRITC-DNP$_{2.3}$-POL	0.1 μg/ml	55.2 ± 11.0
TRITC-DNP$_{2.3}$-POL	1 μg/ml	54.8 ± 10.1
TRITC-DNP$_{2.3}$-POL	30 μg/ml	32.0 ± 3.7
TRITC-DNP$_{3.5}$-POL	0.1 μg/ml	27.4 ± 4.4
TRITC-DNP$_{3.5}$-POL	1 μg/ml	26.5 ± 3.0
TRITC-DNP$_{3.5}$-POL	30 μg/ml	24.2 ± 4.8

TABLE II. Mobility of Receptor-Bound FITC-DNP-DEX on DNP-Specific B Cells

Antigen	Concentration	Percent fluorescence recovery after photobleaching	Diffusion constant (10^{-11} cm^2/sec)	Mobility reduction[a]
FITC-DNP$_{0.24}$-DEX	32 μg/ml	46.9 ± 5.7	18.8 ± 0.35	1
FITC-DNP$_{0.66}$-DEX	33 μg/ml	50.3 ± 8.5	19.0 ± 0.50	1
FITC-DNP$_{1.2}$-DEX	33 μg/ml	47.7 ± 8.9	16.2 ± 0.39	1
FITC-DNP$_{1.2}$-DEX	1 μg/ml	51.4 ± 12.2	18.7 ± 0.56	1
FITC-DNP$_{3.2}$-DEX	33 μg/ml	48.5 ± 15.4	7.5 ± 0.22	3
FITC-DNP$_{3.2}$-DEX	1 μg/ml	48.9 ± 12.3	9.4 ± 0.37	2
FITC-DNP$_{4.8}$-DEX	33 μg/ml	27.4 ± 4.0	2.1 ± 0.11	10
FITC-DNP$_{4.8}$-DEX	1 μg/ml	29.6 ± 13.0	2.4 ± 0.08	9
FITC-DNP$_{8.0}$-DEX	33 μg/ml	16.7 ± 6.1	1.5 ± 0.05	14
FITC-DNP$_{8.0}$-DEX	1 μg/ml	23.6 ± 5.7	1.3 ± 0.02	17
FITC-DNP$_{9.0}$-DEX	33 μg/ml	22.7 ± 7.6	1.2 ± 0.02	18

[a]Reduction of mobility of bound antigen relative to TRITC-DNP$_{0.5}$-MON at 30 μg/ml.

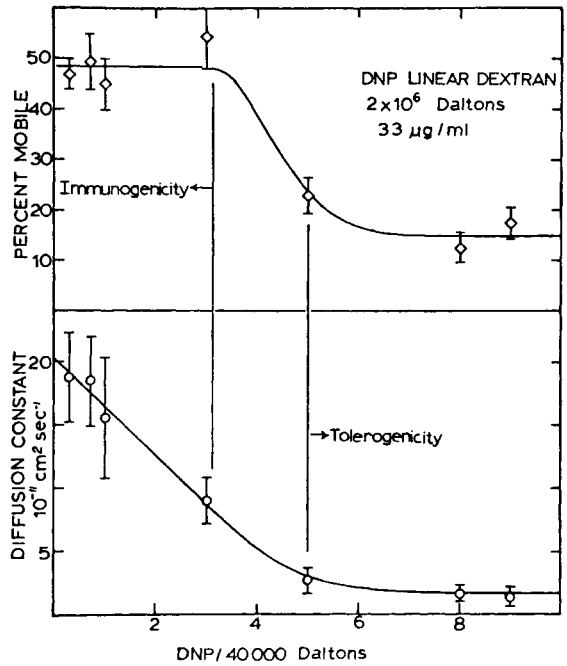

Fig. 5. Top: Fraction of receptor-bound DNP-dextran mobile in photobleaching recovery experiments as function of antigen epitope density. Bottom: Diffusion constant of receptor-bound DNP-dextran as function of antigen epitope density.

hand, active tolerogenicity appears only when the mobility of bound antigen reaches the much smaller value of 2.5×10^{-11} cm^2 sec^{-1}. Finally, one common characteristic shared by POL and DEX antigens is the large difference in the fraction of antigen–receptor complexes mobile in immunogenic and tolerogenic conditions. As with POL antigens already described above, the mobile fraction of FITC-DNP-DEX-receptor complexes fall abruptly from around 50% in immunogenic conditions to below 30% in those cases where the epitope density–dose combination is tolerogenic (Fig. 5a, Table II).

The mobility of bound DNP-POL antigens is not enhanced by cell treatment with colchicine or cytochalasin B, and the mobility is not dependent upon the duration of labeling. These observations suggest that binding of these antigens to the B cell surface is an equilibrium situation. Moreover, the primary determinant of receptor–antigen aggregate mobility does not seem to be interactions with intact microfilaments or microtubules.

DISCUSSION

The details of how epitope density and dose of an antigen may specify delivery of immunogenic or tolerogenic signals varies among different models of B cell activation. In this presentation we are concerned with the following questions: 1) How do epitope density and concentration dependences of antigen–receptor mobilities on the target cell membrane compare between two TI antigens, DNP-POL and DNP-DEX? 2) What is the relationship between mobilities of antigen–

receptor complexes and immunogenicity and tolerogenicity of these antigens? 3) What is the relationship between diffusion constants and sizes of receptor–antigen aggregates on the cell membranes?

Quantitative comparisons of epitope density effects between the two antigens are of little interest since we do not know how conventional epitope density of those antigens relates to the spatial frequency of *accessible* antigenic determinants along the carrier backbones. Nevertheless, it is clear for both antigens that an increase in epitope density results in a large reduction in the mobility of the receptor-bound antigens on the cell membrane. In extreme cases, a large fraction of once mobile receptor–antigen complexes becomes immobile in the time scale of the experiment. These two antigens, however, show very different patterns of epitope density-dependent mobility reductions. The approximately linear relationship between increasing epitope density and mobility reduction index observed with POL antigens is absent for DEX antigens. Instead, a sharp fall of the mobility of bound antigen is observed within a relatively narrow epitope density range.

Reasons why bound antigen mobilities might depend upon antigen dose and epitope density can be found in the physical nature of the binding process. One can reasonably assume that, as increasing numbers of cell surface receptors become incorporated in a receptor–antigen aggregate, the friction constant of the aggregate will increase to some extent and its diffusion constant will therefore decrease. As epitope density increases, a given antigen molecule can and, on the average, will bind more cell surface receptors and so exhibit reduced mobility. The concentration dependence of bound antigen mobility is somewhat more complex. A polyvalent antigen molecule bound to a cell at low external antigen concentrations will in general possess both some free antigenic determinants and some receptors bound to the antigen by only one site, their other site being free. Such an antigen–receptor complex thus presents both free antigenic determinants and free receptor sites and can interact with another such structure to form a larger and slower-diffusing aggregate. Such interactions are favored by increased concentration of cell-bound antigen. As the external antigen concentration rises, so also does the surface concentration of bound antigen. The fraction of bound antigen involved in the multiantigen complexes must therefore increase, and the resulting larger average size of a receptor–antigen complex leads to lower antigen diffusion constants.

That the mobile fraction of bound antigen decreases with increasing antigen epitope density and dose could result in two ways from the above behavior. On the one hand, the distribution of antigen among aggregates of various sizes could be shifted to include very large aggregates diffusing too slowly to appear mobile. Alternatively, aggregates of some critical size or structure might undergo cooperative interactions with cytoskeletal or membrane structures and thus be immobilized.

Quantitative models have been devised for the equilibrium interactions of polyvalent antigens with bivalent cell surface receptors. These models might suggest possible explanations for some of the differences between DNP-POL and DNP-DEX. The most recent treatment applicable to DNP-POL [7], ie, to a rigid antigen, is a relatively straightforward equilibrium thermodynamic description of the process outlined in the previous paragraphs. This treatment draws on DeLisi and Perelson's combinatorial analysis [23] of the possible ways aggregates involv-

ing varying numbers of antigen molecules and receptors can be formed in solution and on Perelson's more recent treatment [9] of the binding of single antigen molecules to the cell surface. The qualitative results of the treatment are that, given the plausible thermodynamic parameters, the sizes of receptor–antigen aggregates increase with antigen dose and epitope density. Another result of this treatment is that monogamous bivalency of receptors, that is, the binding of both sites of a receptor to determinants on the same antigen molecule, may be quite significant.

DNP-DEX presents a much more complicated system. This material is extremely flexible and exists in solution as a random coil. When such a molecule is constrained to bind to a receptor on a cell surface, considerable configurational entropy must be lost, and this unfavorable entropic term opposes such binding. Wiegel and Perelson [9] have devised a theory for the binding of single flexible antigen molecules to a cell surface. Models that permit cross-linking of flexible antigens by bivalent receptors and thus describe antigen concentration-dependent binding have yet to be described. Wiegel and Perelson's calculations [9] indicate that, below a critical antigen epitope density, the unfavorable entropic effects predominate, and binding does not occur. As the antigen epitope density increases above the critical value, the molecule rapidly becomes bound to the surface at more and more points. Initially, when binding is via isolated receptors well separated on the cell surface, the cross-linking is termed "unrestrictive" and is believed to be nonimmunogenic. At higher epitope densities, strings of adjacent antigenic determinants here and there along the antigen bind receptors, each string or "train" bringing several receptors into physical proximity. Such binding is termed "restrictive" and is believed to deliver an immunogenic signal through such possible mechanisms as conformational changes in receptors, activation of membrane-bound enzymes, or the opening or closing of ionic conductance channels. Individual trains existing under these conditions correspond conceptually to the "immunon" of Dintzis et al [24] postulated by these authors to be the fundamental unit of immunogenicity. Finally, at still higher epitope densities, virtually every determinant along the polymer binds receptors in one enormous train producing "overlyrestrictive" cross-linking and tolerance.

Two of our experimental observations suggest that DEX flexibility may indeed be important in determining its mode of interaction with the cell surface. The first of these is the difficulty we have observed in labeling DNP-specific cells with FITC-DNP-DEX of epitope densities below 1. DNP-POL labels cells strongly at much lower concentrations and epitope densities. This may reflect the existence of a critical epitope density for DNP-DEX binding as predicted by Wiegel and Perelson [9]. Second, we find no measurable antigen concentration dependence of DNP-DEX mobility. Our calculations for DNP-POL [7] suggest that monogamous bivalent interactions of receptors with antigen molecules may be important for rigid antigens. For a *flexible* antigen, the possibility that both sites of a given receptor could find antigenic determinants of the same antigen would be greatly increased. Such behavior would greatly decrease the ability of flexible antigens to form multiantigen complexes, and hence would strongly decrease the dose dependence of bound antigen mobility.

There is no simple relationship between the mobility of diffusible antigen–receptor aggregate and immunogenicity–tolerogenicity. This is clearly demonstrated by observations that the diffusion constant of bound POL antigens under condi-

tions of optimum immunogenicity is very similar to those observed under tolerogenic conditions. Moreover, this diffusion constant is manyfold lower than that observed for DEX antigens at the similar immunogenic condition. On the other hand, there is a strict correlation, within the available FPR data, between the fraction mobile and immunogenicity and tolerogenicity for both antigens. In all immunogenic cases, the fraction mobile is around 50–60% compared to 20–30% in tolerogenic cases. It would appear from these observations that, although some restriction of receptor mobility is beneficial or perhaps required for B cell activation, too much restriction (ie, immobilization) results in a tolerogenic response. These observations are similar to those observed in mast cell activation [25] as well as lectin activation of lymphocytes [26]. These observations are also consistent with Perelson's prediction [9] and with the notion that excessive cross-linking of receptors by multivalent antigens indexes immunological tolerance [27, 28].

There are too many gaps in our knowledge of interactions of sIg molecules with the surrounding membrane environment to establish clearly the relationship between observed diffusion constant and aggregate size. Furthermore, although we were unable to confirm such in these studies, there is increasing evidence to suggest that cytoskeletal structures are involved in the modulation of surface protein mobilities [29]. Any attempt to establish the relationship just mentioned must therefore at the present time be regarded with considerable skepticism. Nonetheless, these particular TI antigens are very long polymers—eg, POL rods may be 4,000 Å long. At least for small numbers of receptors bound to a given POL rod, the individual receptors might well be far enough apart to exhibit independent hydrodynamic behavior. This would mean that each receptor contributed to the aggregate friction constant the same value it would in the absence of the other bound receptors. Interactions between receptors and the cytoskeleton need not be negligible; they need only be independent of aggregate size. Finally, calculations suggest that the friction constant of a POL rod or DEX coil in water is negligible relative to that of even a single sIg molecule in the cell membrane. If all these assumptions were met, the diffusion constant of cell-bound antigen would then be proportional to the number of receptor molecules cross-linked and would in fact equal the index of mobility reduction described above. Realistically, however, this simplistic relation between aggregate size and diffusion probably breaks down for at least two reasons. First, the lateral diffusion of a membrane protein is almost certainly determined by interactions with cytoskeletal structures or with other membrane proteins. These interactions need not be simply additive for the different components of extended structures. Second, a Saffman-Delbrück style treatment [30] of the hydrodynamics of such diffusion is necessary, and such treatment must include new features. In particular, the object or array must be variably permeable to the medium. This has recently been accomplished for cylindrically symmetric objects by Wiegel [31]. Laterally extended objects must, however, be considered, and this has not yet been done.

Whatever relation may exist between the sizes and diffusion constants of receptor–antigen aggregates, the data on DNP-DEX make a particularly interesting point. If one compares the immunogenicity data in Figure 3 with the antigen-diffusion constants in Table II and Figure 5, it is clear that DNP-DEX exhibits significant immunogenicity when the mobility of bound antigen differs hardly at all from that of a single sIg molecule. Under these conditions, most an-

tigen molecules must be bound to a single cell-surface receptor. In such a case Wiegel and Perelson's theory [9] predicts that the fractions of antigen molecules bound to more than one receptor must fall off strongly with the numbers of receptors cross linked. Thus the immunogenicity of such low epitope density flexible antigens must derive from a small number (perhaps 10%) of antigens cross-linking pairs of sIg molecules or from the *exceedingly* small number of antigens cross-linking much larger numbers of receptors. In neither case can most of the sIg be involved in aggregates of 12–16 receptors. Aggregates of this size have been postulated [24] as the fundamental unit of immunogenicity. Our data suggest that, if the immunogenicity of flexible TI antigens does indeed require formation of receptor-antigen aggregates of this size, then *very* few such aggregates per cell are sufficient to deliver an immunogenic signal.

We certainly do not wish to oversimplify the process of B cell activation. We recognize that antigen-specific B cells may receive necessary additional signals from, say, macrophages. Moreover, B cell activation involves multiple steps occupying a much longer time frame than we have examined here. On the other hand, antigens do certainly interact with B cell membrane receptors under conditions that lead to antibody and/or proliferative responses. This interaction can and must be studied as an independent physical phenomenon before the impact of other signals can be assessed.

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