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Immunology

Specific cellular stimulation in the primary immune response: Experimental test of a quantized model

(T cell-independent antigen/dinitrophenyl-substituted polyacrylamide/B cell-receptor linkage/"immunon" theory/ immune response and suppression)

R. Z. DINTZIS[†‡], B. VOGELSTEIN[§], AND H. M. DINTZIS[‡]

[†]Department of Cell Biology and Anatomy, [‡]Department of Biophysics, and [§]Oncology Center, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

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ABSTRACT **Dose–response and dose–suppression curves have been measured for the primary immune response in mice, *in vivo* and *in vitro*, by using size-fractionated linear polymers of acrylamide substituted with hapten. The results are in general agreement with a simple theory based on the premise that the specific primary immunological response is quantized at some fundamental and limiting step, requiring a minimum number of linked antigen receptors for response.**

The immune response to most antigens is a very complex phenomenon that has resisted quantitative analysis. Some of this complexity may be related to the molecular complexity of many of the antigens (e.g., proteins, cell walls, etc.) which have been used. A relatively simple and specific primary immune response occurs against antigens that are linear polymers of only a few types of chemical subunits (1–3). These antigens give rise to a rapid primary immune response with the production of specific antibody molecules predominantly of the class IgM (4, 5). The immune response to these antigens may be relatively simple because of its rapidity and the apparent minimum involvement of thymus-derived lymphocytes (T cells). Although complex interactions involving suppressor cells, helper cells, and idiotypes seem to be important in more mature, T cell-dependent responses, it is likely, as discussed here, that they are not a dominant factor in the very early and rapid T cell-independent responses.

It long has been observed that multivalent antigens are better immunogens than are monovalent ones; indeed, a number of investigators have proposed that triggering of immunocompetent cells (B cells) to form antibody requires adequate cross-linking of surface receptors by multivalent ligands (6–14). We have carried this important observation one step further by proposing that there is a specific number of receptors that must be linked together in order to deliver the triggering signal (15). The interaction site between antigen and receptor often has been proposed as occurring on the surface of B lymphocytes (16). Various kinds of antigen-presenting cells also may play a role at this site (17, 18); however, for the sake of simplicity, we have taken the limiting step to be one occurring on the external surfaces of B cells.

We have described the preparation of and the *in vivo* immunological response to a series of size-fractionated linear polymers of acrylamide partially substituted with dinitrophenyl (Dnp) hapten (15), which behaved as "T cell-independent" antigens. Analysis of the responses elicited by a series of such polymer preparations led us to the conclusion that the primary immunological response at some basic and limiting level is quantized—i.e., a minimum specific number of antigen receptors (which we estimated to be ≈12–16) must be connected together as a spatially compact cluster, an "immunon," before an immunogenic signal is delivered to the receptor cell.

The present paper gives the results of using size-fractionated Dnp-polyacrylamide preparations in two kinds of experimental procedures: (*i*) measurement of the shape of the dose–response curve as a function of the dose of polymer as administered *in vivo* and *in vitro* and (*ii*) assessment of the inhibitory effect on the response to immunogenic polymers caused by the presence of polymers that are not substituted with enough haptens to be immunogenic. These results are compared with the predictions of a general theory of primary immune responsiveness to T cell-independent immunogens that we have developed (19). The theory, which is based on the immunon model of immune responsiveness, predicts several phenomena: (*i*) a requirement for a minimum number of spatially linked haptens in order for a polymer preparation to be immunogenic (15); (*ii*) the occurrence of a predicted shape of dose–response curve for a homogeneous immunogenic polymer preparation; and (*iii*) the occurrence of a predicted shape and location of dose–suppression curve with increasing doses of nonimmunogenic polymer. We believe that the data presented here show a remarkable quantitative concordance between theory and experiment.

SUMMARY OF IMMUNON THEORY

The immunon model assumes that (*i*) each cell capable of responding to a haptenated T cell-independent immunogen contains a large number of individual hapten receptor molecules on its membrane surface; (*ii*) close spatial clustering of these receptors results from their sequential binding to appropriately spaced haptens on one immunogenic molecule; (*iii*) an immunon can be formed, but only very slowly, when the receptor cluster contains the critical number of linked receptors (q, the immunon number); (*iv*) the cell will receive a specific stimulus, when sufficient "immunons" have been formed, that initiates a complex and multistep process leading to cell division, cellular differentiation, and antibody production; and (*v*) the amount of the primary immune response that is induced in an animal not previously exposed to the hapten is directly related to the rate of immunon formation in the population of cells bearing receptors for that hapten.

The kinetic process of immunon formation is symbolized in Fig. 1 in which receptors on a cell surface are shown to be interacting with a molecule of immunogenic (or stimulatory) polymer S (Fig. 1*a*) or interacting with a molecule of nonimmunogenic (or nonstimulatory) polymer N (Fig. 1*b*). Polymer N, which we have shown (15) is not capable of causing a specific

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Abbreviation: Dnp, dinitrophenyl.

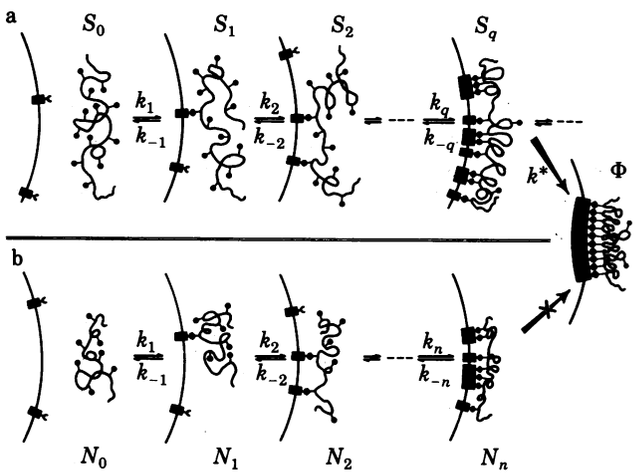

FIG. 1. A diagrammatic representation of the stepwise assembly of an immunon cluster, Φ. The solid rectangles represent mobile protein receptor molecules containing binding sites (cups). The curved solid lines represent flexible polyacrylamide molecules with attached Dnp groups shown as solid circles. Receptors are shown as reacting stepwise either with an immunogenic polymer, *S*, or with a nonimmunogenic polymer, *N*. Subscripts on *S* and *N* are used to designate polymer molecules bound to clusters of surface receptors of the specific number designated by the subscript. Polymer *N* cannot form immunons because it cannot simultaneously bind enough receptors (because $n < q$).

immune response at any dose because it has an insufficient number of hapten groups, has been found to inhibit strongly the immunogenic effects of polymer *S*. According to our model, the inhibition is caused by nonproductive competition for binding sites. The essential difference between the immunogenic molecule *S* and nonimmunogenic molecule *N* is that the former can bind at least q cell receptor molecules, whereas the latter cannot (where q is the immunon number). The model assumes that once q cell receptors have been bound, the molecular cluster represented by S_q can undergo a slow, irreversible structural transformation with rate constant k^* to form immunons. As shown in an accompanying paper (19), theoretical consideration of the differential equations describing the formation of immunons leads directly to a quantitative relationship expressing the immune response as a function of the concentration of immunogenic and nonimmunogenic molecules.

From this relationship it has been shown (19) that if doses D_S of immunogen and D_N of nonimmunogen are injected into one animal and doses D'_S and D'_N are injected into a second animal, then the ratio r of immune response in the first animal relative to that in the second animal should be given by

$$r = \frac{D_S}{D'_S}\left[\frac{(q-1)\,D_S^{\max} + D'_S + D'_N}{(q-1)\,D_S^{\max} + D_S + D_N}\right]^q \qquad [1]$$

where $D_S^{\max}$ corresponds to the dose of immunogen giving maximum response in an animal—i.e., the peak of the dose–response curve. The peak of the curve corresponds to optimal occupancy of receptor groups by large molecular clusters (19). Addition of more immunogenic polymer causes a decrease in the average cluster size. Addition of nonimmunogenic polymer competes nonproductively for receptor sites. In either case, immunon formation is inhibited by nonproductive competitive inhibition. Thus, high-dose suppression by immunogenic polymer and suppression by nonimmunogenic polymer both operate by a common mechanism—competitive inhibition of immunon formation by nonproductive binding of specific receptors.

MATERIALS AND METHODS

Mice. BALB/c female mice were obtained from the Charles River Supply, Wilmington, MA. CAF_1 (BALB/c × A) female mice were obtained from The Jackson Laboratory. Mice were 6–8 weeks old when immunized.

Polymer Preparations. The preparations of linear polyacrylamide substituted with Dnp hapten groups used in this study were derived from those used in a previous study (15) by further column fractionation on Sepharose CL-4B. Preparation *N* was a central subfraction of polymer *B*, previously shown to be nonimmunogenic (not stimulatory) and preparation *S* was a central subfraction of polymer *D*, previously shown to be immunogenic (stimulatory). Measurement of partial specific volume (0.690 ml/g) and extrapolation of sedimentation equilibrium molecular weight to zero concentration gave values of 60,000 for *N* and 130,000 for *S*. These values together with dry weight and absorbance at 360 nm show *N* to contain 19 Dnp groups per molecule [7–9 "effective" or appropriately spaced (15)], whereas *S* contains 43 Dnp groups per molecule (14–21 "effective"). Polymers *N* and *S* have almost identical "epitope densities" or degrees of substitution by hapten per molecular size unit.

Antibody Response. Polymer preparations were injected intraperitoneally in 0.5 ml of isotonic saline. After 6 days, blood was collected by bleeding from the tail, and the serum was stored at −30°C until analysis. The concentration in serum of IgM antibody against Dnp was determined by a solid-phase binding assay (unpublished work). Surfaces covalently coated with Dnp-substituted gelatin served to bind the anti-Dnp mouse antibody, whose presence was then measured by a second incubation with I^{125}-labeled rabbit antibody against mouse IgM antibody supplied by Robert Johnson (20).

***In Vitro* Culture and Assay.** Mice were killed by cervical dislocation, and their spleens were minced in RPMI-1640 medium and pressed through a stainless steel mesh (60 × 60 mesh; 0.019-cm diameter). Cellular debris was allowed to settle, and the supernatant containing a dispersed-cell suspension was decanted, freed of erythrocytes by osmotic shock, and washed. Suspensions of nucleated spleen cells were then incubated with or without appropriate polymer in 60 × 15 mm tissue culture dishes containing 5×10^7 viable cells in a final volume of 7.5 ml. The incubation was carried out in 5% CO_2/95% water-saturated air at 37.0°C. The incubation medium consisted of RPMI 1640 medium enriched with 5% (vol/vol) heat-inactivated fetal calf serum, 2% (vol/vol) heat-inactivated horse serum, 4 mM glutamine, 100 units of penicillin and 100 μg of streptomycin per ml, and 50 μM 2-mercaptoethanol.

After 3 days of incubation, cells were harvested and washed. Assay for direct (IgM) anti-Dnp plaque-forming cells was performed by using a modification of the procedure of Jerne *et al.* (21).

RESULTS AND DISCUSSION

The immunological response in BALB/c mice 6 days after injection of various doses of immunogenic polymer preparation *S*, as measured by the concentration of serum IgM molecules reactive toward Dnp groups, is shown in Fig. 2. The mice in this experiment came in a single shipment of uniform age from the supplier and were divided into groups of 10. Members of each group were injected with the same dose, and all groups were handled as uniformly as possible. The solid curve in Fig. 2 is the theoretical response curve expected from Eq. 1 as visually fitted to the experimentally determined points by adjustment of the numerical value of $D_S^{\max}$ to 0.3 μg. In view of the simplicity of the assumptions involved in the derivation of Eq. 1 and the known variability of response of individual mice,

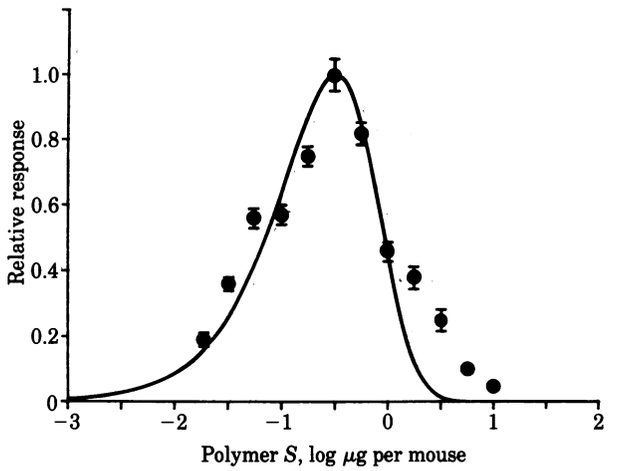

FIG. 2. Dose–response measurements showing the mean of the relative concentration, in serum from individual mice, of IgM antibody against Dnp at 6 days after injection of polymer *S* in amounts shown (10 BALB/c mice per point). Error bars indicate SEM when it is larger than the circle. The solid curve gives the theoretical response expected from Eq. 1 for a peak response occurring at a dose of 0.3 μg per mouse and an immunon size, *q*, of 10. The theoretical response is not sensitive to the value of *q* if *q* is greater than five. The peak of the response curve corresponds to approximately 30 μg of anti-Dnp IgM per ml of serum.

the agreement between theory and experiment is surprisingly good. However, when the experiment was repeated by using different groups of mice supplied by the same breeder, the variability of biological responses in whole animals became more evident. Fig. 3 compares the dose–response curves of three separate shipments of BALB/c mice and illustrates both group-dependent variability of response of individual mice at each dose and some change of shape of the dose–response curve from group to group. The variable immunological response given by different groups of mice is a well-known phenomenon (22, 23), having been observed both in studies using whole animals and in those using cell cultures. It probably is dependent on factors in the previous history and handling of the animals, such as exposure to bacteria, viruses, and parasites, which might influence the "antigenic naivete" of the animals, as well as exposure to environmental shocks such as heat and cold during shipment.

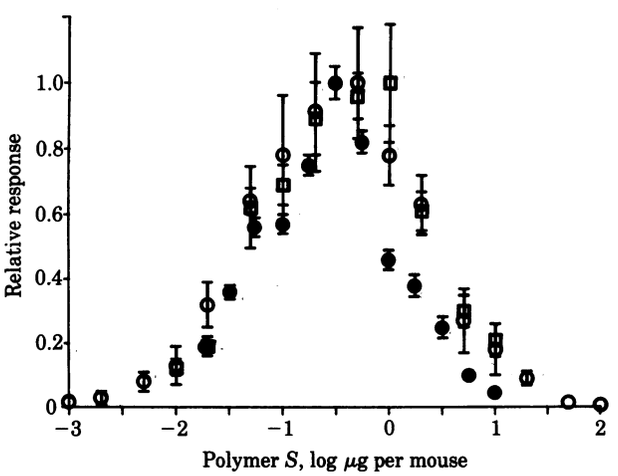

FIG. 3. Dose–response measurements for different lots of BALB/c mice. Measurements were made on serum from individual mice. The mean of measurements on each group at each dose is shown, together with the SEM when it is larger than the symbol. ●, Ten mice per point (these points are the same as in Fig. 2); ○, five mice per point; □, six mice per point.

By comparing the observed dose–response curves shown in Figs. 2 and 3 with the theoretical curve shown in Fig. 2, it is clear that although the agreement between curves is good, the observed responses are quite variable from one batch of mice to another and, in general, show a wider dose–response curve than expected from the simple model that generated the curve shown in Fig. 2.

We explain the wider experimental curve in the following way. The theoretical curve in Fig. 2 is based on the assumption that all cells responding to the immunogen have receptor molecules with the same binding constant for Dnp groups. This assumption of complete homogeneity is unlikely to be true. If cells that bind immunogen and respond to it have protein receptors with differing binding constants for Dnp, then the predicted response should be the sum of a number of individual cellular response curves. Each curve would be like that in Fig. 2, but those with lower binding constants would be displaced to the right by an amount proportional to the ratios between their binding constants for Dnp. Inspection of Figs. 2 and 3 from this point of view indicates that the observed width of the experimental dose–response curves may be understood as resulting from the summation of responses from individual populations of cells having receptors differing in binding constants by 1–1.5 log units—i.e., 10- to 30-fold. The dose–response measurements can be fit within experimental error by summing the theoretical responses of three or four such populations (19).

For a constant dose of immunogenic polymer, Eq. 1 also predicts the extent of reduction of response that will be obtained with doses of increasing amounts of nonimmunogenic polymer *N*. Measurements of this type are shown in Fig. 4 for BALB/c mice. The solid line in Fig. 4 is not fitted to the data but is calculated directly from Eq. 1 by using the estimated value of the maximum-response dose, D_S^{max}, of 0.5 μg per mouse obtained from Fig. 3. The agreement between the experimental points and the calculated theoretical curve in Fig. 4 seems remarkable, if we consider the absence of arbitrarily adjusted parameters in this calculation.

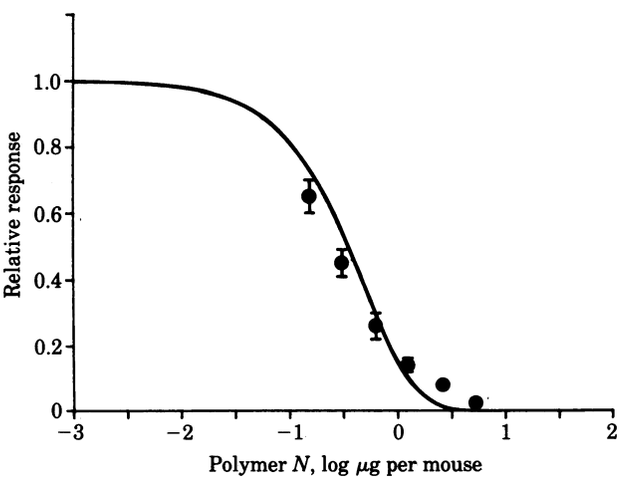

FIG. 4. Response-reduction measurements for increasing doses of nonimmunogenic polymer preparation *N* injected simultaneously with a constant dose of imunogenic polymer preparation *S*. Measurements were made on serum from individual mice. The mean of each group is shown together with the SEM when it is larger than the symbol. BALB/c mice, 10 mice per point; 0.31 μg of polymer *S* given to each mouse. The solid curve gives the theoretical response expected from Eq. 1 for an immunon size, *q*, of 10 and D_S^{max} set equal to 0.5 μg per mouse as derived from Fig. 3. The theoretical response is quite insensitive to the value of *q* but is shifted left or right according to the value of D_S^{max}, with no change in shape.

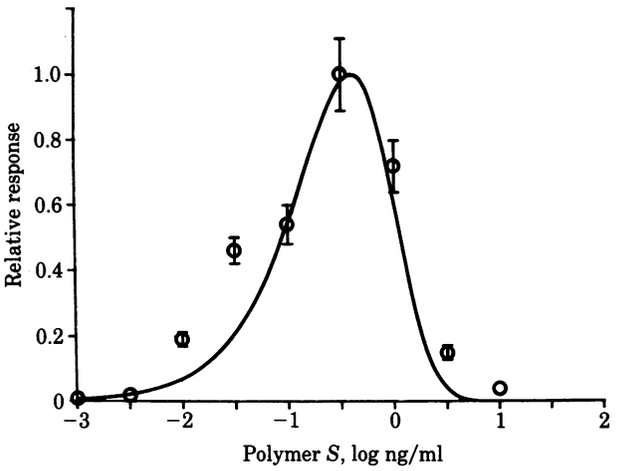

FIG. 5. Dose–response measurements showing the relative number of direct anti-Dnp plaques produced from spleen cell cultures 3 days after the start of incubation in the presence of various concentrations of immunogenic polymer *S*. The data represent the mean of duplicate cultures with triplicate assays per culture; the SD is indicated when it is larger than the circle. The experimental peak response corresponds to ≈300 plaques per 10^6 spleen cells with a blank (without polymer) of ≈20 plaques per 10^6 spleen cells. The solid curve gives the theoretical response expected from Eq. 1 for a peak response occurring at a polymer concentration of 0.4 ng/ml and an immunon size, *q*, of 10.

In addition to experiments in living animals shown in Figs. 2, 3, and 4, dose–response curves were measured *in vitro* with isolated mouse spleen cells. Fig. 5 shows the results of such an *in vitro* experiment as compared with a visually fitted theoretical curve calculated from Eq. 1. The agreement between experiment and theory for the *in vitro* experiment with cultured spleen cells (Fig. 5) is approximately as good as it was for the *in vivo* experiment with whole mice (Fig. 2). In both cases, the measured response curve is somewhat broader than that predicted from a model based on a homogeneous hapten binding constant in the responding cells (see above).

Measurements of the inhibition of immune response *in vitro* with increasing amounts of nonimmunogenic polymer are shown in Fig. 6. The solid line is not fitted to the data but is

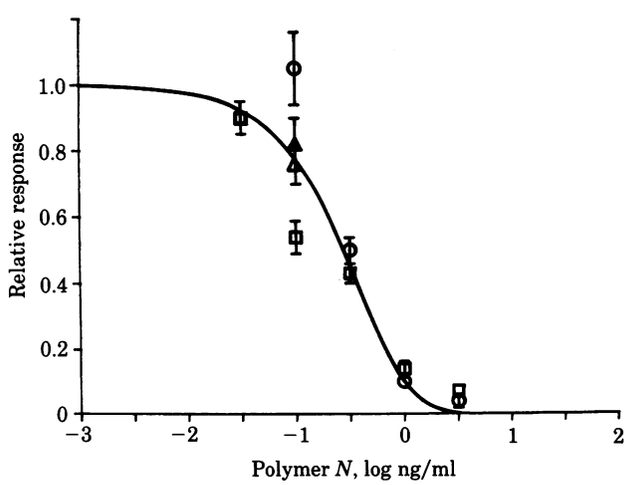

FIG. 6. Dose–reduction measurements for increasing doses of nonimmunogenic polymer preparation *N* incubated in spleen cell culture with a constant dose (0.3 ng/ml) of immunogenic polymer preparation *S*. Procedures and data treatment were as in Fig. 5. The different symbols show data obtained in separate experiments. The solid curve gives the theoretical response expected from Eq. 1 for an immunon size, *q*, of 10 and D_S^{max} set equal to 0.4 ng/ml as derived from Fig. 5.

calculated directly from Eq. 1 by using the estimated value of the maximum-response dose, D_S^{max}, of 0.4 ng/ml from Fig. 5. The agreement between the experimental points and the calculated theoretical curve is quite good, again in the absence of arbitrary parameters.

The blood volume and extracellular fluid volume of a mouse are each ≈1 ml, so the optimal immunogenic polymer dose *in vivo* is ≈1 μg/ml. There is a large apparent discrepancy between this *in vivo* dose and that which is optimally immunogenic *in vitro* (≈1 ng/ml). The almost 1000-fold sensitivity difference is largely explained by rapid removal *in vivo* of polymer molecules by phagocytes located throughout the body. Studies with ^{125}I-labeled preparations of the polymers as described (15) showed that the bulk of the injected polymer is quickly removed from the circulation by Kupffer cells in the liver and phagocytic cells in other tissues (unpublished results). The resulting rapid fall in free polymer concentration, coupled with uncertainties concerning the rate of equilibration of polymer between different body fluid compartments makes difficult any quantitative comparison of relative optimum concentrations *in vivo* and *in vitro*. In spite of these difficulties, there remains the fact that the shapes of the dose–response and dose–suppression curves measured *in vivo* are remarkably similar to those measured *in vitro*, implying strongly that the same limiting process is being probed in both cases. Furthermore, in both cases the measured responses as a function of dose are in excellent agreement with the predictions of Eq. 1.

Although polymer *N* fails to stimulate at any dose (15), it inhibits polymer *S* at the same dose where polymer *S* is maximally stimulatory. This implies a competition for surface receptors. Because both polymer preparations have almost identical "epitope densities" with a common carrier chemistry, this finding is in disagreement with theories that explain immunogenicity by invoking epitope density (2) or polyclonal (i.e., nonspecific) activation by the "carrier" (24, 25).

In interpreting the above data, we wish to postulate several points concerning the nature of a specific T cell-independent stimulus: (*i*) a specific immunogenic signal is generated by the formation of immunons on the surface of a responsive cell, (*ii*) an immunon will form only after a sufficient number of surface receptors are clustered, and (*iii*) specific clustering of surface receptors occurs as a consequence of their being bound to linked haptens. This binding is specific for the hapten–receptor interaction and does not primarily depend on the "scaffolding" to which the haptens are attached. The underlying physical scaffold that links the haptens may be molecular in nature or may consist of a surface on which small hapten-containing structures are aggregated, as on the surface of an "antigen-presenting cell."

Nonspecific stimuli, such as mitogens, lectins, antibodies against cell surface proteins, and activating or inhibiting factors from other cells, may well influence the level of "irritability" of the responding cell, making it more or less likely to respond to a given amount of immunogenic signal or even to respond in the absence of specific signals. Factors from T cells (26, 27) and macrophages (28, 29) have been shown to enhance antibody responses nonspecifically. Mitogens are known to stimulate cells nonspecifically to secrete antibodies (25, 30). Whether or not they do this directly or indirectly by a mechanism involving specific receptor aggregation is not known. However, in contrast to these nonspecific stimuli, we postulate that specific stimulation occurs by means of the linkage of receptors by their specific binding sites into immunons; thus, cells displaying those receptors are stimulated to divide and differentiate into cells that will secrete specific antibodies.

Current immunological theory focuses much attention on

regulation of the immune response by networks of suppressor and helper cells operating through idiotype–anti-idiotype networks (31, 32). Our experimental data suggest that it is not necessary to invoke either of these phenomena to explain the early and rapid response to a T cell-independent antigen, Dnp-polyacrylamide. Therefore, we propose that such networks are not dominant effects under the conditions of our study. At later times or with T cell-dependent antigens, or both, it is likely that they are substantial or even dominant components of immune regulation. Our data likewise do not permit us to differentiate between short-term suppressive effects and longer-term tolerogenic effects (33) of inhibitory polymers.

We have demonstrated that molecules consisting of haptens linked to a flexible linear polymer are immunogenic only if they have a sufficient number of adequately spaced haptens. This finding with a T cell-independent antigen might at first seem contradictory to the fact that many protein molecules that are T cell-dependent antigens and which do not contain multiple identical antigenic sites are nevertheless antigenic. However, several studies have shown that the antigenicity of proteins *in vivo* depends on their state of aggregation (34). It is well-known that experimentally induced aggregation of protein molecules by physical methods (heat, adsorption to bentonite, emulsification with Freund's adjuvant) (35) or by chemical methods (cross-linking with glutaraldehyde or alum) (35) greatly enhances their antigenicity. Nonaggregated protein molecules centrifuged free of aggregates or collected from the sera of injected animals have been shown to be not immunogenic but tolerogenic, whereas aggregated material with presumed multiple antigenic sites produces an immune response (36, 37). Therefore, it seems quite possible that the minimum requirements for antigenicity as determined with our simple T cell-independent polymer may have applicability to immune responses to a large variety of molecules, including T cell-dependent ones.

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