

ANTIGEN VALENCE DETERMINES THE BINDING OF
NOMINAL ANTIGEN TO CYTOLYTIC T CELL CLONES

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In general the specificity of T lymphocytes is such that the nominal antigen used to generate a particular T cell response does not bind directly to the relevant T cells. Instead it is recognized in the context of self major histocompatibility complex (MHC) gene products expressed on the surface of an antigen-presenting cell. Recently (1, 2), two groups have isolated T cell clones of the helper phenotype that can bind nominal antigen directly, but it remains unclear why the binding of nominal antigen to T cells has been detected only in a few instances. We have initiated studies with defined synthetic antigens to determine which structural parameters permit a given nominal antigen to bind directly to antigen-specific, MHC-restricted T cell clones.

Our approach is based on the notion that low affinity binding of nominal antigen might be detected using highly multivalent antigens containing a sufficient number of appropriately spaced antigenic determinants. Such antigens should bind to multiple receptors on the T cell surface, thereby enhancing the overall avidity of the interaction. We chose the hapten fluorescein isothiocyanate (FL) as a nominal antigen and prepared a series of water-soluble, FL-conjugated polymers varying in backbone chemistry, antigen valence (number of FL groups per molecule), epitope density (number of FL groups per unit of polymer length or mass), and net molecular weight.¹ The binding of these polymers to FL-specific murine cytolytic T cell (CTL) clones was then assessed by flow cytometry. Initial studies showed that high molecular weight (600,000 to 2,000,000 [600–2,000 K]) polymers conjugated with 200–800 FL groups per molecule bound in a highly specific fashion to anti-FL CTL clones and induced lymphokine secretion by the clones.¹

In this paper we examine the role of antigen valence in the binding of nominal antigen to CTL clones. We show that the stable binding of FL-conjugated polymers to FL-specific CTL clones occurs only if the valence exceeds a certain critical number. Molecules with a lower valence do not give detectable binding. This result may help to explain discordant results obtained in previous studies of antigen binding to T cells.

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Materials and Methods

Reagents. The I isomer of FL was obtained from Research Organics, Inc., Cleveland, OH. The hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was synthesized as previously described (3). Linear polyacrylamide (PA) was synthesized from crystalline acrylamide (5). Ficoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ and fractionated as described below.

Polymers. Starting PA preparations of appropriate molecular weight were isolated by gel filtration. Fractions with average molecular weights of 40 and 600 K were obtained by chromatography of high molecular weight PA on Sepharose CL-2B columns as previously described (4, 5). Columns were run in a buffer consisting of phosphate-buffered saline (PBS), 0.1 mM EDTA, and 0.02% (wt/vol) NaN_3 .

Polymer preparations were conjugated with FL as follows. Amino groups were introduced into PA as previously described (4, 5) and into Ficoll according to the procedure of Inman (6). Amino groups were conjugated to FL at pH 9.2 in 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$. The polymers were then dialyzed exhaustively against gel filtration column buffer.

After conjugation, polymer preparations were fractionated by gel filtration, taking narrow cuts to achieve a relatively narrow molecular weight distribution. The 40 K mol wt fraction of substituted PA (PA40) was then rechromatographed on a 90 cm Sepharose CL-6B column, and the 600 K mol wt fraction of substituted PA (PA600) was rechromatographed on a 90 cm Sepharose CL-2B column. Center cuts were taken to ensure a relatively homogeneous molecular weight distribution. The 40 K mol wt Ficoll fraction (Fic40) was derived from a commercial Ficoll preparation with an average molecular weight of 70 K (Ficoll 70; Pharmacia Fine Chemicals) by taking a low molecular weight gel filtration cut on a Sepharose CL-6B column after conjugation to FL. The 700 and 2,000 K mol wt Ficoll fractions (Fic700, Fic2000) were derived from a commercial preparation with an average molecular weight of 400 K (Ficoll 400; Pharmacia Fine Chemicals) by taking high molecular weight gel filtration cuts on a Sepharose CL-2B column after conjugation to FL.

Aliquots of these fractions were then dialyzed exhaustively against distilled water. FL content was determined by measuring optical density at 493 nm in 0.01 M $\text{Na}_2\text{B}_4\text{O}_7$ vs. dry weight. Molecular weights were determined by sedimentation equilibrium analysis in the analytical ultracentrifuge as previously described (4, 5). Measurements were performed at several polymer concentrations using the short column method, and molecular weights were obtained by extrapolation to zero polymer concentration. The characteristics of the polymer preparations used in this study are given in Table I.

Coupling of FL and NIP to Stimulator and Target Cells. Cells were covalently coupled with FL by incubation at 2×10^7 cells/ml with 0.5 mM FL in saline-borate buffer, pH 9.0, for 15 min at 37°C. Cells were covalently coupled with NIP as described (3).

Generation of Hapten-specific CTL Clones. CTL clones specific for target cells conjugated

TABLE I
Characteristics of FL-conjugated Polymers

Designation	Polymer backbone	Average molecular weight* ($\times 10^{-5}$)	Antigen valence (FL groups/molecule)	Epitope density (moles FL/gram polymer) ($\times 10^4$)	Average [†] spacing \AA	Binding
FL ₃₄₀ PA600	PA	6.0	340	5.67	61	+
FL ₂₁ PA40	PA	0.4	21	5.25	68	-
FL ₆₀₀ Fic2000	Ficoll	20.0	600	3.00	— [‡]	+
FL ₁₄ Fic40	Ficoll	0.4	14	3.50	—	-
FL ₂₂₅ Fic700	Ficoll	7.0	225	3.21	—	+
FL ₈₇ Fic700	Ficoll	7.0	87	1.24	—	-

* Average molecular weights were determined by sedimentation equilibrium analysis as described in Materials and Methods.

[†] Determined by assuming that the approximate length of a monomer subunit within an extended acrylamide polymer equals 2.5 Å.

[‡] The crosslinked structure of Ficoll precludes a direct calculation of average spacing.

with the haptens FL and NIP were established as described elsewhere.¹ Briefly, B6D2F₁/J mice were immunized by weekly intraperitoneal injections of 10⁷ mitomycin C-treated, hapten-derivatized stimulator cells. After 3–5 wk, spleen cells from immune mice were cultured with hapten-coupled stimulator cells in RPMI 1640 supplemented with glutamine, pyruvate, nonessential amino acids, antibiotics, 2-mercaptoethanol (5 × 10⁻⁵ M), and 10% fetal calf serum (FCS). Beginning on day 4, cultures were supplemented with 10 U/ml of interleukin 2 (IL-2), prepared as previously described (3). On day 15, responding cells were cloned by limiting dilution at 0.3, 1, 3, and 10 cells per well in Terasaki plates. Clones with hapten-specific cytolytic activity were selected and expanded in the presence of 10 U/ml IL-2. Fresh stimulator cells were added weekly. Cells to be used in binding experiments were grown in the absence of FL-conjugated stimulator cells for at least 14 d.

⁵¹Cr Release Assays. Assays were done using lymphoblast targets as described (3).

Binding Experiments. Cells were incubated at 1–2 × 10⁷ cells/ml with indicated concentrations of FL-conjugated polymers in the presence of the metabolic inhibitors 2-deoxy-D-glucose (50 mM) and NaCN (1 mM). This combination of inhibitors greatly suppresses the internalization of fluid phase markers by endocytosis (7), without affecting cell viability in short-term incubations. Since binding plateaus after ~30 min at 20°C,¹ an incubation time of 1 h was used. After incubation, cells were washed extensively and processed for flow cytometry.

Flow Cytometry. Binding was analyzed by flow cytometry on a fluorescence-activated cell sorter (FACS II; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Raw data from all test samples were gated to remove dead cells and aggregates from the analysis. Scatter gates were set using samples stained with 1.5 μg/ml propidium iodide to stain dead cells. Data from FACS experiments are presented as histograms in which relative cell number is plotted against fluorescence channel number or as plots of the mean fluorescence channel number as a function of another parameter. The standard errors of the mean fluorescence channel number values were always ≤0.5 channels.

Results

To study the binding of nominal antigen to CTL clones, we isolated from B6D2F₁ mice a series of FL-specific, MHC-restricted CTL clones (termed BD-FL-1, BD-FL-2, and BD-FL-3). These clones were selected on the basis of their ability to lyse FL-conjugated syngeneic target cells.¹ A syngeneic NIP-specific clone (BD-NIP-1) was used as a control in binding experiments. The ability of these clones to cause hapten-specific lysis of syngeneic targets is shown in Fig. 1.

We have shown that FL-specific clones will specifically bind high molecular weight (600–2,000 K) polymers conjugated with 200–800 FL groups per molecule and that this binding induces lymphokine secretion by the clones.¹ To examine the structural parameters of the antigen that are important in the binding reaction, we first compared the binding of polymer preparations with the same epitope density or spacing between FL groups but different molecular weights (and therefore different valences). FL- and NIP-specific clones were incubated with equivalent weight concentrations (10 μg/ml) of FL₃₄₀PA600 or FL₂₁PA40. These linear polymers have approximately the same spacing of FL groups but differ in molecular weight by a factor of 15. Neither polymer bound to the NIP-specific control clone BD-NIP-1 (Fig. 2 A). FL₃₄₀PA600 bound to BD-FL-1, BD-FL-2, and BD-FL-3. However, FL₂₁PA40 did not bind to any of these clones, even at a 15-fold molar excess. Similar results were obtained with Ficoll polymers. Fig. 3 shows dose response curves for the binding of FL₆₀₀Fic200 and FL₁₄Fic40 to BD-NIP-1 and BD-FL-1. Again, neither polymer bound well to the NIP-specific control clone. FL₆₀₀Fic2000 bound to BD-FL-1, but FL₁₄Fic40, which has a slightly higher epitope density, failed to bind. Thus, for

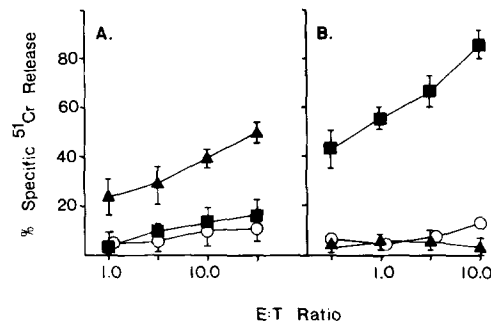


FIGURE 1. Hapten specificity of cytotoxicity by anti-NIP and anti-FL CTL clones. Clones BD-NIP-1 (A) and BD-FL-1 (B) were tested on syngeneic lymphoblast targets that were mock-derivatized (○) or derivatized with NIP (▲) or FL (■). E:T, effector/target ratio.

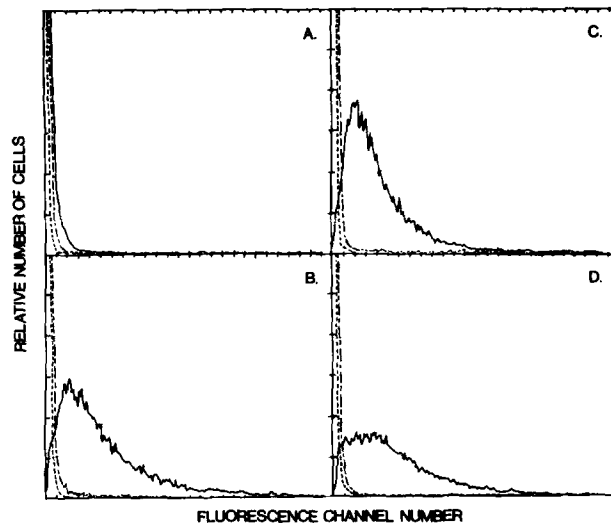


FIGURE 2. Gated fluorescence histograms for cells incubated with medium alone (---), 10 $\mu\text{g}/\text{ml}$ FL₂₁PA40 (---), or 10 $\mu\text{g}/\text{ml}$ FL₅₄₀PA600 (—). (A) Control clone BD-NIP-1; (B) BD-FL-1; (C) BD-FL-2; (D) BD-FL-3. Cells were incubated with 10 $\mu\text{g}/\text{ml}$ of polymer for 1 h at 20°C and then washed and analyzed by flow cytometry. 20,000 cells were analyzed per sample. Voltage and gain settings were the same for each sample.

two different FL-conjugated polymers, large molecules bind well while small molecules with the same epitope density do not. This result suggests that the total number of epitopes per molecule, or the antigen valence, may be a critical factor in the stable binding of nominal antigen to T cell clones. Because larger molecules bound while smaller ones did not, we next investigated the possibility that size alone, irrespective of antigen valence, might determine binding. We compared the binding of FL₂₂₅Fic700 and FL₈₇Fic700, two polymers with the same molecular weight but a different number of epitopes per molecule. FL₂₂₅Fic700 bound specifically to BD-FL-1 but FL₈₇Fic700 did not (Fig. 4). Neither polymer bound to the control clone, even at the high dose of 300 $\mu\text{g}/\text{ml}$. Thus, molecular size alone does not determine binding. Rather, it appears that the number of epitopes per molecular or the antigen valence is a critical factor in determining the binding of nominal antigen to antigen-specific CTL.

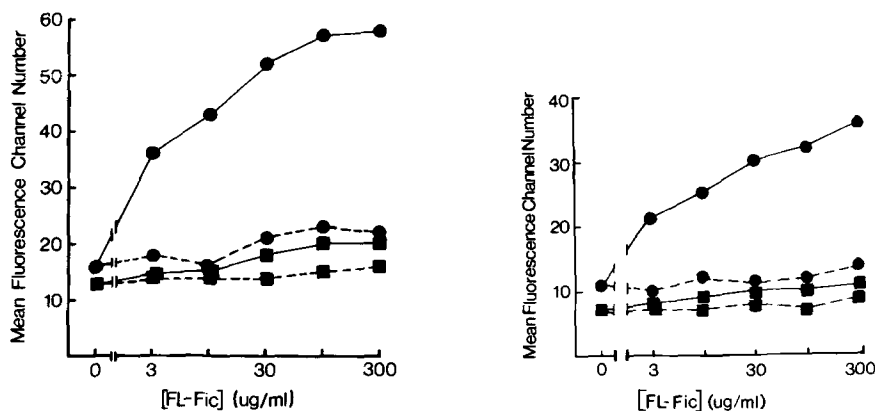


FIGURE 3. Comparative dose-response curves for the binding of FL₁₄Fic40 (---) and FL₆₀₀Fic2000 (—) to BD-NIP-1 (■) and BD-FL-1 (●). Cells were incubated with the indicated concentration of polymer for 1 h at 20°C and then washed and analyzed by flow cytometry. 10,000 cells were analyzed per sample. Voltage and gain settings were the same for each sample.

FIGURE 4. Dose response curves for the binding of FL₈₇Fic700 (---) and FL₂₂₅Fic700 (—) to BD-NIP-1 (■) and BD-FL-1 (●). Cells were incubated with the indicated concentrations of polymer for 1 h at 20°C and then analyzed by flow cytometry. 10,000 cells were analyzed per sample. Voltage and gain settings were the same for each sample.

Discussion

We have investigated the parameters required for detectable binding of nominal antigen to antigen-specific CTL clones. Binding of 600–2000 K mol wt polymers conjugated with >225 FL groups per molecule was readily detectable by flow cytometry analysis. In contrast, 40 K mol wt polymers with fewer epitopes per molecule (but the same epitope density) failed to bind. A large 700 K mol wt polymer with only 87 FL groups per molecule also failed to bind. Taken together, these results suggest that a certain critical number of receptor-ligand bonds are required to produce an attachment that is stable enough to be detected under these conditions. Since FL₂₂₅Fic700 bound readily to BD-FL-1, an upper limit for this critical number of bonds is 225. However, the actual number of bonds formed is probably substantially less than 225, since not all of the FL groups on a given polymer are both sterically accessible and spaced far enough apart from adjacent FL groups to permit binding to separate receptors. For linear polymers such as FL₂₁PA40, all of the FL groups should be accessible. However, by assuming that the T cell receptor complex has a diameter of 100 Å, we estimate (using an exponential distribution function [5]) that somewhat less than half (~10) of these FL groups will be spaced far enough apart from other FL groups to permit binding to independent receptors. Since FL₂₁PA40 did not show detectable binding, the minimal number of effective FL groups per molecular required for stable binding is between 10 and 225. A series of polymers with intermediate valences are being prepared to determine this number more precisely. The number may vary for different clones and different antigens. For example, Rao et al. (2) detected antigen binding to arsonate-specific helper T cell clones using proteins conjugated with six to nine arsonate groups per molecule, although the binding of aggregated antigen was not ruled out. In any

event, our results suggest that a high degree of multivalence is required for the stable binding of nominal antigen to certain CTL clones.

The simplest interpretation of these data is that the intrinsic association constants for individual T cell receptor-antigen interactions are extremely low. Such weak interactions are expected to have rapid dissociation rates, making them difficult to detect unless the ligand is held near the receptor in some manner. Multivalent binding can produce this kind of tethering effect, resulting in an enormous enhancement of the avidity of the interaction (8–10). Since the binding of nominal antigen to T cells is detectable only if the antigen is present in a highly multivalent form, the intrinsic association constants of individual T cell receptor-antigen interactions may be much lower than those of other physiologic receptor-ligand interactions for which the binding of monovalent ligands to cell surface receptors is readily detectable.

Summary

We have shown that cytotoxic T cell clones specific for the nominal antigen FL will bind high molecular weight (600,000 to 2,000,000) polyacrylamide and Ficoll polymers conjugated with 200–600 FL groups per molecule. Low molecular weight polymers (40,000) with the same epitope density did not give stable binding. A high molecular weight polymer with a lower epitope density also failed to bind. Taken together, these results suggest that a substantial degree of multivalence is a necessary factor in the stable binding of nominal antigen to T cell clones.

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