Inhibition or Activation of Human T Cell Receptor Transfectants Is Controlled by Defined, Soluble Antigen Arrays

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Summary

We present evidence that direct T cell receptor (TCR) occupancy by antigen can either activate or inhibit T cells, depending upon whether or not a threshold number of local TCRs are crosslinked by multivalent arrays of the antigen. Variants of Jurkat cells were previously transfected with TCR α and β chains that bind fluorescein, yielding FL-TCR$^+$ human T cells. The transfectants are activated upon binding soluble multivalent antigen arrays at concentrations well below those required for monovalent interactions. This activation, measured by calcium fluxes and interleukin 2 (IL-2) production, indicates the superior binding avidity of multivalent ligands. Smaller, less multivalent arrays do not activate the cells, but antagonize larger arrays, demonstrating that antigen can bind TCR as either agonist or antagonist. The balance between activation and inhibition depends upon antigen array size, ligand valence, and concentration, indicating that a threshold extent of receptor crosslinking, and not individual perturbations of single TCR, is required for activation by antigen. Approximately 100 stimulatory arrays specifically bind per FL-TCR$^+$ cell at concentrations where IL-2 production is half-maximal.

T cells are activated by antigen fragments bound to MHC gene products on APCs (1). Specific T cell activation is mediated by TCR α and β chains, which have dual specificity for antigens and MHC proteins (2–6). Interactions between TCRs and TCR ligands (either MHC–peptide complexes or anti-TCR Abs) result in a cascade of events, including phosphatidylinositol metabolism and calcium fluxes, protein phosphorylation and dephosphorylation, and coordinate immediate early gene activation, lymphokine secretion, cytokine receptor expression, and cellular proliferation (7, 8).

As noted recently, "the key question is whether T cell triggering involves crosslinking of the TCR molecules, or whether activation occurs by perturbation of a TCR multi-molecular complex by a single MHC-peptide" (9). It has long been known that anti-TCR Abs or peptide–MHC complexes alone can activate T cells when immobilized in highly multivalent form on beads, presenting cells, or planar membranes (10, 11). However, the valence of antigen and the number of crosslinked TCRs required for activation has remained unclear, because quantitation of TCR interactions with such solid phase ligands has remained experimentally difficult (12–14).

To begin to answer this question, several laboratories have recently prepared homogeneous, soluble TCR antigens (MHC–peptide complexes) and TCRs, which have revealed that monovalent TCR–ligand interactions are of low affinity ($K_d \sim 10^{-5}$ to 10^{-6} M; 15–18). To overcome these low affinities of monovalent TCR-ligand binding, both interactions with accessory molecules, and multivalent TCR–MHC peptide interactions, have been proposed to explain the exquisite specificity for antigen by T cells (17–19).

It remains unclear, how TCR ligation by nominal antigen on APCs is related to TCR crosslinking by anti-TCR Abs, although both signals trigger qualitatively similar biochemical responses (1). In an effort to bridge this important gap, we have used an experimental system where hapten antigen binds TCR α and β heterodimers directly, without ligation of MHC or accessory proteins and without resulting synergistic and/or antagonistic effects (15, 20, 21). It is notable that other hapten-specific T cells that bind antigen in the absence of MHC proteins have also been described, although most TCRs bind peptide–MHC complexes (1, 22–25).

We chose to study the function of soluble fluorescein antigen arrays because they could be systematically varied with respect to valence and physical size. Such a range of valences has not yet been studied using either soluble MHC–peptide complexes or anti-TCR antibody. Additionally, the study of

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multimolecular intercellular interactions in T cell activation would be simplified by varying the defined, soluble antigen arrays as the sole experimental variable. Our study differs from previous work in that: (*a*) soluble, multivalent antigen, rather than antibodies directed against TCR epitopes, was studied; (*b*) antigen arrays were systematically varied to include a wide range of valences that have not been studied with peptide–MHC complexes or antibody Fab fragments, to analyze requirements for TCR crosslinking by antigen; (*c*) soluble, radiolabeled arrays were used, facilitating direct quantitation of binding (without fluorescence quenching); and (*d*) correlations were made between direct antigen binding and T cell function.

Some of the soluble antigen arrays activate T cells at concentrations far below the K_d of monovalent TCR–antigen interactions (15), which indicates that multivalent TCR-ligand binding can indeed occur with great avidity (i.e., effective affinity), despite such weak monovalent TCR-ligand interactions. We report below that direct TCR occupancy by multivalent antigen can inhibit or activate T cells, which strongly suggests that a threshold number of local TCRs must be coordinately ligated (crosslinked) for activation of calcium fluxes and IL-2 production. Furthermore, the results suggest that single TCR–antigen interactions do not trigger such T cell functions.

Materials and Methods

Cells, Culture Media, and IL-2 Assay. Jurkat cells and fluorescein (FL)[1]-TCR$^+$ transfected Jurkat cells (derived from TCR β^- Jurkat 31–13 cells; [15, 21]) were grown and assayed in complete medium, using Eagle's MEM, suspension salts (Gibco Laboratories, Grand Island, NY) supplemented with nonessential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, essential amino acids, 1 g/liter dextrose, and 10% (vol/vol) FCS (Hyclone Laboratories, Inc., Logan, UT) as described (2). To maintain long-term episomal expression of TCR in stable transfectants, geneticin and hygromycin were each added to complete medium at 0.5 mg/ml (21). For IL-2-assay, T cells were cultured overnight in 0.3 ml complete medium in flat-bottomed microculture plates (Costar, Cambridge, MA), pelleted by centrifugation at 250 *g* for 10 min, and 0.1 ml sterile culture supernatants were removed and incubated overnight with IL-2–dependent CTLL-2 cells. The proliferation of IL-2–dependent cells was assessed by measuring [^{3}H]thymidine incorporation after overnight incubation with 1 μCi [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) using a PhD cell harvester (Cambridge Technology, Inc., Cambridge, MA) and glass fiber filters (Schleicher & Schuell, Inc., Keene, NH) as described (26).

Synthesis, Purification, and Characterization of TCR Ligand Arrays. Fluorescein arrays were prepared as previously described (27), by derivatizing size-fractionated polyaminoethyldextran, polyaminoethylficoll, or polyaminoethylpolyacrylamide with fluorescein isothiocyanate (isomer I; Sigma Chemical Co., St. Louis, MO, or Molecular Probes, Eugene, OR) or other fluorophores. Excess fluorescein was removed by exhaustive dialysis. The mass of antigen arrays was determined after dialysis against deionized water and drying under vacuum to constant weights. A characteristic value for refractive index per mass was determined for each array chemistry, using a variety of reference solvents. The molecular weight distribution of purified antigen arrays in aqueous solution was determined after fractionation in 0.1 M potassium phosphate buffer containing 20% (vol/vol) acetonitrile, pH 8, on tandem Superose 6 and Superose 12 fast protein liquid chromatography (FPLC) columns (Pharmacia, Piscataway, NJ; and HP-1090 liquid chromatograph; Hewlett-Packard Co., Palo Alto, CA), by measuring light scattering and refractive index using a Dawn F laser photometer and Wyatt Optilab 903 refractometer (Wyatt Technology Corp., Santa Barbara, CA) (28). Ligand valence was determined by spectrophotometry at 496 nm using a molar extinction coefficient of 72,000 M^{-1} (diode array spectrophotometer model 8451A; Hewlett Packard).

Intracellular Calcium Measurements. FL-TCR$^+$ transfectants were loaded with 2.5 μM Indo-1 AM (Molecular Probes, Eugene, OR) for at least 30 min at 37°C. Single cell intracellular calcium measurements were determined at 37°C as described, by measuring the ratio of emissions at 410 and 490 nm upon excitation at 370 nm using a flow cytometer (Coulter Electronics Inc., Hialeah, FL) and Cicero software (Cytomation Inc., Fort Collins, CO) (29). As a positive control, the ratio of emissions was determined for cells treated with anti-TCR antibody (2Ad2A2, kindly provided by Dr. R. Siliciano, Johns Hopkins) or ionomycin. No PMA was added during any of the calcium measurements.

Radiolabeled Arrays and Binding Experiments. Fluorescein arrays were diluted in 0.1 M potassium phosphate buffer, pH 8, to 2 × 10^{-4} M fluorescein, 0.5 mCi Na^{125}I (Dupont-New England Nuclear, Boston, MA) was added in 50 μL of the same buffer, and the mixture incubated with an Iodobead (Pierce Chemical Co., Rockford, IL) for 30 min at 23°C on a nutating platform. Trace-labeled arrays (with <1/100 fluoresceins iodinated) were separated from free Na^{125}I using a 10 ml desalting column (Speedy; Pierce Chemical Co.) preequilibrated in PBS. Specific activity of the iodinated arrays was determined by measuring radioactivity and OD_{496}. Cells were incubated at 4°C with various concentrations of radiolabeled $Flu_{960}Dex_{2300}$, with or without excess identical unlabeled competitor overnight, and then were washed at least three times through ice-cold FCS by centrifugation at 4°C and aspiration of supernatants using a 30-gauge needle. Radioactivity of cell pellets was determined. The number of arrays bound per cell was calculated, using the specific activity of the arrays and assuming quantitative recovery of cells.

Results

FL-TCR$^+$ T Cells Are Specifically Activated by Soluble Antigen Arrays, Resulting in Bell-shaped Dose Response Curves. T cell clones that proliferate in response to soluble fluorescein arrays have been isolated, and their TCR α and β chains previously were transfected into a TCR β^- variant of Jurkat cells, yielding FL-TCR$^+$ T cells (21). Jurkat cells are a widely used human tumor cell line that mimics resting peripheral human T cells. These cells secrete IL-2 upon incubation with a combination of soluble anti-TCR antibody and PMA, as part of the multifaceted processes of T cell activation (7).

To confirm that the transfected cells specifically and directly are activated by fluorescein antigen, Jurkat cells or FL-TCR$^+$ transfectants were incubated with PMA at 3 ng/ml and various concentrations of a physically defined, soluble fluorescein array with ~3,600 fluoresceins per 10^7 Da mass dextran polymer backbone. We assayed T cell activation by

[1] *Abbreviation used in this paper:* FL, fluorescein.

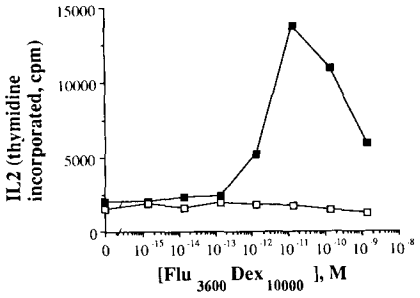

Figure 1. Soluble antigen polymers specifically activate FL-TCR$^+$ transfectants. Comparison of IL-2 production by Jurkat cells (□) vs. FL-TCR$^+$ transfectants (■) incubated with soluble fluorescein array. Cells were cultured at 2 × 10^5/ml with indicated concentrations of $FL_{3,600}Dex_{10,000}$ kD and with PMA added at 3 ng/ml. Culture supernatants were harvested and assayed for IL-2 concentration using IL-2–dependent CTLL-2 cells. As control, maximum [^{3}H]thymidine incorporation by CTLL-2 cells with exogenous IL-2 was 380,000 cpm; without added IL-2, CTLL-2 cells incorporated 1,500 cpm. Similar results were obtained in six separate experiments.

measuring IL-2 production. T cell culture supernatants were harvested and thymidine incorporation was determined using IL-2–dependent cells (CTLL-2 [26]). As shown in Fig. 1, untransfected Jurkat cells did not produce IL-2 when treated with PMA and these soluble fluorescein-dextran arrays at any concentration. However, FL-TCR$^+$ transfectants, which bear T cell antigen receptors that specifically bind fluorescein in the absence of MHC gene products (21), produced IL-2 when so treated. Moreover, FL-TCR$^+$ transfectants were not stimulated under similar conditions by arrays bearing Texas red or rhodamine, two structural analogs of fluorescein (D. Symer, unpublished data). The results verify that FL-TCR$^+$ cells, unlike the parental Jurkat cells which express different TCR α and β chains, are activated by fluorescein arrays specifically (21).

FL-TCR$^+$ Jurkat transfectants produced IL-2 in response to anti-TCR antibody and PMA under conditions similar to those previously described as activating the parental Jurkat cells (7). The total secreted IL-2 was about 50% less by FL-TCR$^+$ cells than by parental Jurkat cells, at similar doses of anti-TCR antibody, consistent with lower levels of total TCR expression by the former cells (21).

A biphasic pattern of increasing and then decreasing T cell activation, measured by IL-2 production, is observed with increasing antigen array and constant PMA concentrations (Fig. 1). Such a "bell-shaped" dose–response relationship has previously been observed with increasing TCR antigen or with increasing MHC gene product concentrations (30), although the reasons for these effects with soluble vs. surface-bound TCR ligands may be different. A possible mechanism accounting for our findings (Fig. 1) is that, at high doses, the antigen arrays would be toxic to cells. In three separate experiments, we have observed no significant difference in proliferation of cells treated with fluorescein arrays even at 10^{-4} M fluorescein moiety concentrations, ruling out cell death (apoptosis) as a cause of decreased IL-2 production at high antigen concentrations.

Another possible mechanism that would account for these bell-shaped dose–response curves is that thresholds of TCR multimerization must be exceeded for T cell activation. At high concentrations of TCR ligands such as the soluble antigen arrays, individual arrays would not crosslink a threshold number of neighboring TCRs to activate the T cell, because of competition between the numerous such arrays binding neighboring TCRs. According to this model, smaller and less multivalent arrays would not activate T cells, because they would not crosslink enough neighboring TCRs at any con-

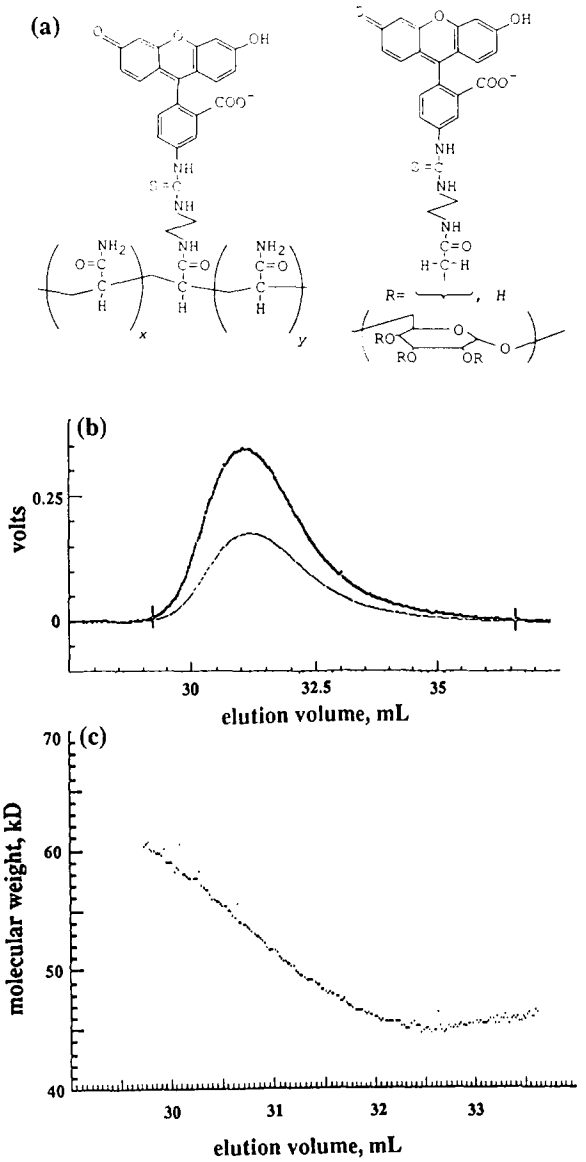

Figure 2. Physical properties of antigen polymers. (*a*) Chemical linkages of fluorescein isothiocyanate, isomer I, to polyacrylamide (*left*) and to dextran or Ficoll (*right*). (*b*) Fractionation of a representative fluorescein-Ficoll array sample in aqueous solution, by HPLC gel filtration. Voltage signals indicating 90° laser light scattering (*heavy line*) and refractive index (*thin line*) are shown as a function of its elution volume. (*c*) Molecular weight distribution of the array sample as a function of its elution volume, calculated from laser light scattering and refractive index (28).

centration. To test whether individual perturbations of single TCR by antigen would or would not trigger cell activation, we synthesized and characterized a series of related antigen arrays.

Physical Characteristics of Soluble Antigen Arrays. Related, soluble antigen arrays were synthesized, using various carrier molecules of different size and chemistry, and bearing different numbers of fluorescein ligands. The chemical linkages of fluorescein-polyacrylamide and fluorescein-dextran arrays, used below, are depicted in Fig. 2 *a*. These ligand arrays were purified to relative homogeneity as described (20, 27). Similar ligand arrays activate and/or inhibit both T-independent and high affinity, T-dependent IgG and IgE immune responses in vivo (31; Symer, D., J. Reim, S. Schneider, R. Z. Dintzis, and H. M. Dintzis, unpublished data).

Using a new method, we characterized physical sizes and ligand valences of the arrays in solution by measuring laser light scattering, refractive index, and optical density. The measured 90° light scattering and refractive index of a typical antigen array sample, fractionated by HPLC gel filtration, are shown in Fig. 2 *b*. Based on such measurements, the calculated molecular weight distribution for the eluted sample in solution is shown in Fig. 2 *c*. Clearly, the molecular weights of individual array molecules comprising this sample are not identical, and range from 45 to 60 kD. The mean molecular mass for the arrays of the sample is 53 kD, and >95% of the array molecules are within 15% of the mean. Antigen valence was determined by measuring optical density for samples of known mass and average molecular weight as described (27). We found that about 12 fluoresceins are on the 53-kD Ficoll backbones, and so this relatively homogeneous sample is designated as $Flu_{12}Fic_{53}$. A summary of the average molecular weights and valences of antigen arrays used in this paper, determined as shown in Fig. 2, *b* and *c*, and/or by sedimentation equilibrium as described (27), is presented in Table 1.

Table 1. *Defined, Soluble Antigen Arrays*

Name	Backbone	Fluorescein valence	Average array size
			Da
$Flu_{12}Fic_{53}$	Ficoll	12 per array	53,000
$Flu_{20}Fic_{96}$	Ficoll	20	96,000
$Flu_{50}Fic_{200}$	Ficoll	50	200,000
$Flu_{260}Fic_{770}$	Ficoll	260	770,000
Flu_8Dex_{21}	Dextran	8	21,000
$Flu_{960}Dex_{2300}$	Dextran	960	2,300,000
$Flu_{3600}Dex_{10000}$	Dextran	3,600	10,000,000
$Flu_{150}PA_{630}$	Polyacrylamide	150	630,000

Summary of average sizes and ligand valences of antigen arrays. Average values were calculated using data as shown in Fig. 2. Ligand valence was determined by measuring OD at 496 nm. Concentrations of arrays were determined by measuring dry weights of representative samples as described (27).

Activation of FLTCR$^+$ T Cells Is Determined by Array Size and Ligand Valence. To test the proposed model as an explanation for observed bell-shaped dose–response curves in T cell activation, we tested the relative efficacy of antigen arrays that were varied with respect to ligand multiplicity and carrier mass (at approximately constant ligand density). As shown in Fig. 3, two different multivalent array samples, $FL_{50}Fic_{200}$ and $FL_{260}Fic_{770}$, stimulated IL-2 production in a bell-shaped pattern as a function of dose. By contrast, physically smaller arrays of lower ligand multiplicity, i.e., oligovalent $FL_{20}Fic_{96}$ and $FL_{12}Fic_{53}$, did not induce substantial IL-2 production at any concentration. Total input fluorescein and backbone mass concentrations were equivalent for both stimulatory and nonstimulatory arrays (i.e., they were all of approximately constant ligand density). Additionally, bell-shaped dose–response patterns were obtained when large, densely haptenated fluorescein-polyacrylamide or fluorescein-dextran arrays were tested. We have not yet determined what array sizes and valences define the transition between stimulatory and nonstimulatory properties of polyacrylamide carriers.

Smaller, oligovalent arrays such as $FL_{20}Fic_{96}$ and $FL_{12}Fic_{53}$ did not induce substantial IL-2 production at any concentration. The results (Figs. 1 and 3) are consistent with the above model that may account for bell-shaped dose–response curves: the fluorescein multiplicity and/or array size of $FL_{20}Fic_{96}$ and $FL_{12}Fic_{53}$ may not be sufficiently large, resulting in insufficient coordinate ligation of TCR by individual antigen arrays.

Antigen-specific Inhibition of T Cell Activation Using Oligovalent Arrays. It is possible that nonstimulatory arrays, with ligand valence < about 20, did not activate the FLTCR$^+$ cells because they did not bind the cells at all. To test this possibility, we determined whether smaller, oligovalent arrays could inhibit activation by stimulatory arrays. As shown in Fig. 4 *a*, oligovalent FL_8Dex_{21} at any concentration failed to stimulate IL-2 production, confirming the results shown in Fig. 3 with fluorescein-Ficoll, a different chemical backbone. As positive control, $FL_{50}Fic_{200}$ was tested separately at var-

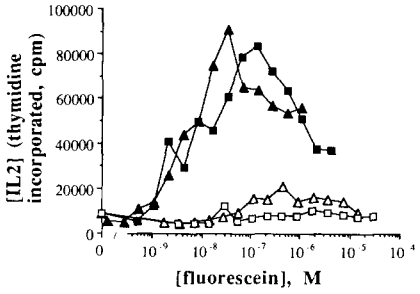

Figure 3. IL-2 production by FLTCR$^+$ transfectants is controlled by array size and valence. Comparison of IL-2 stimulation using antigen arrays of different size and valence. FLTCR$^+$ transfected cells at 3×10^5/ml were incubated with various concentrations of fluorescein-Ficolls of similar ligand density per mass carrier backbone: (▲) $Fl_{50}Fic_{200}$; (■) $Fl_{260}Fic_{770}$; (△) $Fl_{20}Fic_{96}$; and (□) $Fl_{12}Fic_{53}$. Culture supernatants were harvested and assayed for IL-2 content, by measuring [^{3}H]thymidine incorporation by IL-2–dependent cells. Similar results were obtained in at least two separate experiments.

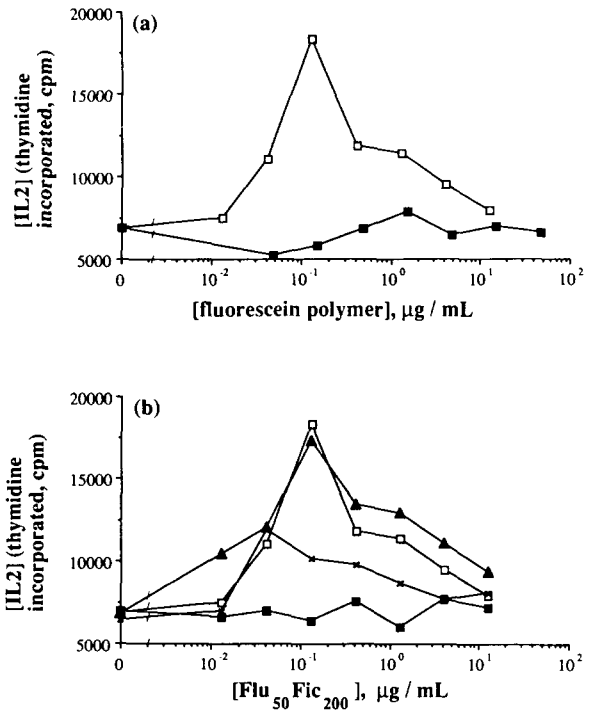

Figure 4. Inhibition of T cell activation using nonstimulatory antigen arrays. (*a*) FL-TcR$^+$ transfectants were cultured overnight at 7 × 10^5 cells/ml with PMA at 3 ng/ml and various concentrations of $FL_{50}Fic_{200}$ (□) or FL_8Dex_{21} (■). Culture supernatants were harvested and assayed for IL-2 by measuring proliferation of IL-2–dependent cells. (*b*) Inhibition of IL-2 production, stimulated as in (*a*) by $FL_{50}Fic_{200}$, using nonstimulatory FL_8Dex_{21}: (□) no added FL_8Dex_{21}; (▲) 0.5 μg FL_8Dex_{21}/ml; (X) 5 μg FL_8Dex_{21}/ml; (■) 15 μg FL_8Dex_{21}/ml. Conditions for IL-2 assay were as in (*a*).

ious doses. We determined whether antigen-specific inhibition of T cell activation occurs when oligovalent antigen is added. As shown in Fig. 4 *b*, various doses of FL_8Dex_{21} were added in combination with stimulatory $FL_{50}Fic_{200}$. The induction of IL-2 by the latter antigen array was inhibited by excess doses of the nonstimulatory array.

Antigen arrays, both oligovalent and multivalent, at epitope concentrations >10^{-4} M fluorescein, are not toxic to FL-TCR$^+$ cells or in the IL-2 bioassay, because transfected cells and IL-2–dependent cells each proliferate normally in their presence as measured by vital dye exclusion and thymidine incorporation (data not shown).

It is notable that the oligovalent array FL_8Dex_{21} added in Fig. 4 is different from the stimulatory $FL_{50}Fic_{200}$ array, in its carrier backbone. Results similar to those in Fig. 4 were obtained when both stimulatory and inhibitory arrays are comprised of the same carrier. Moreover, no inhibition of activation by stimulatory arrays was observed when a vast excess of nonfluoresceinated backbone molecules (i.e., no specific ligands) was added (D. Symer, unpublished data). The results demonstrate that inhibition does not occur by nonspecific competition between carriers per se, because arrays of appropriately low ligand multiplicity can effectively inhibit stimulatory arrays comprised of different carrier chemistry. The results also demonstrate that the oligovalent arrays specifically bind to FL-TCR. Binding sufficient to inhibit activation by more multivalent arrays occurs, even though the more multivalent arrays may bind with greater effective affinity (20).

Inhibition or Activation of Intracellular Calcium Fluxes by TCR Ligand Arrays. As described above, a clear distinction could be made between antigen arrays that induce IL-2 production and those that do not. Arrays having greater than about 100 kD mass and 50 TCR ligands were stimulatory (i.e., multivalent), whereas those with less than about 20 fluoresceins were not (i.e., oligovalent). We tested whether the antigen arrays similarly could be divided according to their efficacy in activating more immediate signals in T cells. Intracellular calcium concentrations rise after only several seconds upon adding crosslinking anti-TCR antibody (7, 32). These rises in intracellular calcium are thought to occur after activation of tyrosine kinases (33, 34).

FL-TCR$^+$ transfectants were loaded with a calcium-sensitive, fluorescent dye, i.e., Indo-1 AM. Intracellular calcium concentrations were determined for individual cells as a function of time by measuring the ratio of fluorescence emissions at two wavelengths, using flow cytometry as described (29). The ratio of violet to blue fluorescence emissions increases as Indo-1 binds increasing intracellular free calcium. As shown in Fig. 5, *a*, *b*, and *e*, the larger antigen polymers $FL_{50}Fic_{200}$ and $FL_{150}PA_{630}$ induced rapid, substantial, dose-dependent rises in intracellular calcium in FL-TCR$^+$ transfected T cells in the absence of PMA. By contrast, different doses of smaller, more oligovalent $FL_{12}Fic_{53}$, which did not induce IL-2 production (Fig. 3), also failed to induce substantial calcium fluxes compared with untreated cells as shown in Fig. 5, *c* and *d*. Furthermore, when $FL_{12}Fic_{53}$ was added at doses where stimulatory arrays were effective, i.e., at iden-

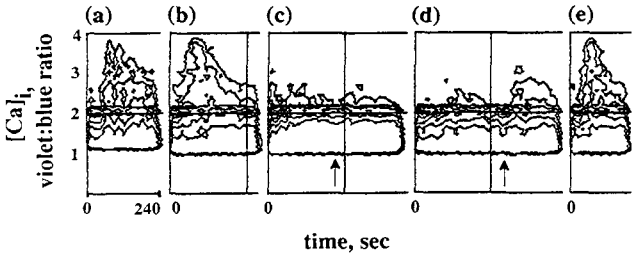

Figure 5. Activation and inhibition of intracellular calcium fluxes by stimulatory vs. nonstimulatory antigen arrays. FL-TCR$^+$ transfected cells at 10^6 cells/ml culture medium were incubated at 37°C with 25 μM Indo-1 AM. After at least 30 min of incubation, intracellular calcium levels were determined for individual cells at 37°C, as a function of time. At time *t* = 20 s, TCR ligands were added at indicated final concentrations: (*a*) $FL_{50}Fic_{200}$ at 2.5 μg/ml; (*b*) $FL_{50}Fic_{200}$ at 25 μg/ml; (*c*) $Fl_{12}Fic_{53}$ at 400 μg/ml followed at *t* = 225 s by $Fl_{50}Fic_{200}$ at 2.5 μg ml; (*d*) $FL_{12}Fic_{53}$ at 400 μg/ml followed at *t* = 300 s by $FL_{50}Fic_{200}$ at 25 μg/ml; (*e*) $FL_{150}PA_{630}$ at 25 μg/ml. Data are plotted as isograms, with outermost contours representing 16 cells/s, then 32, 64, and 128, and were gated for single cells whose emission ratio was greater than or equal to the median ratio, arbitrarily set to 1 (29). This ratio corresponds to $[Ca]_i \sim 100$ nM, whereas a maximal ratio of 4 is ~ 1,000 nM free calcium. No PMA was added in this experiment. Similar results were obtained in three separate experiments.

tical concentrations of fluorescein, it again did not cause a rise in calcium concentrations (D. Symer, unpublished data).

After adding nonstimulatory $FL_{12}Fic_{53}$ and waiting for <5 min, we added stimulatory $FL_{50}Fic_{200}$ at the times indicated by arrows in Fig. 5, *c* and *d*. In both cases, the calcium flux induced by stimulatory polymer (as in Fig. 5, *a* and *b*) was dramatically reduced in most cells (compare *a* and *d*, or *b* and *c*). It is notable that the response to anti-TCR mAb 2Ad2A2 (7) proceeded normally in the presence of oligovalent arrays such as $FL_{12}Fic_{53}$ (D. Symer, unpublished data). This result demonstrates that inhibition of calcium fluxes by oligovalent arrays does not occur by toxicity or by downstream uncoupling of TCR-mediated signal transduction. Furthermore, the time necessary for inhibition is brief, as at most only a few minutes are required for induction of observed suppressive effects.

The resting concentration of free intracellular calcium in Jurkat cells has been reported to be about 100 nM, which would correspond to a normalized ratio of 1 as shown in Fig. 5 (29). Based on this value, we estimate that maximal intracellular calcium, corresponding to a ratio of about 4, is about 1,000 nM free calcium in the activated FL-TCR$^+$ cells.

Estimation of the Number of Antigen Arrays Needed to Activate FL-TCR$^+$ Transfectants. Previous reports have indicated that about 200 TCR ligands (either peptide–MHC complexes or anti-TCR mAbs) on a single APC or bead were necessary to activate IL-2 production by T cells at a minimally detectable level (12, 13), although larger numbers were obtained in a different experimental system (35). To correlate direct binding with T cell function, we sought to determine how many antigen arrays are directly bound to FL-TCR$^+$ cells at concentrations where IL-2 production is half-maximal, because it is difficult to extrapolate to very low concentrations of bound ligands given the inherent heterogeneity of receptor numbers per cell and of cell responses (21, 36).

When fluorescein is bound by different specific mAbs, its fluorescence is variably quenched (37, and Schneider, S., and H. M. Dintzis, unpublished data). Therefore, for unambiguous quantitation of binding, we trace-labeled antigen arrays, with less than one per hundred fluoresceins radioiodinated, to measure binding to FL-TCR$^+$ cells. As shown in Fig. 6 *a*, binding of a large, multivalent, radiolabeled ligand array, which increases with increasing concentration, was substantially reduced by excess identical unlabeled competitor, indicating that binding is specific. As another control for specificity, we added excess unlabeled multivalent fluorescein-polyacrylamide arrays in a different experiment, and again observed substantial competition using the different chemical backbone (data not shown).

Using the specific activity of radiolabeled ligand arrays and the concentrations of FL-TCR$^+$ cells, we calculated the number of specifically bound antigen arrays at various input concentrations from data in Fig. 6 *a*, as shown in Fig. 6 *b*. For comparison, we superimposed results from two independent experiments measuring IL-2 production induced by the same stimulatory arrays at 37°C. In Fig. 6 *c*, we ascertained

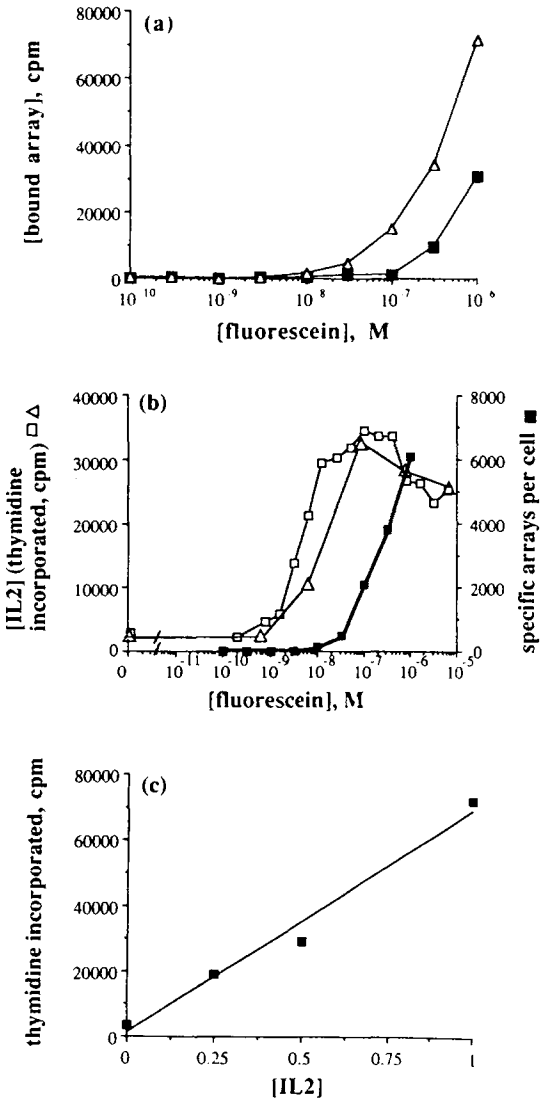

Figure 6. Relationship between antigen array binding and IL-2 stimulation. (*a*) Radioiodinated $FL_{960}Dex_{2300}$ was added at indicated concentrations to FL-TCR$^+$ cells at 1.8×10^6 cells/ml culture medium containing unlabeled excess $FL_{960}Dex_{2300}$ at 10^{-5} M fluorescein (■); or no competitor (△). Cells were incubated at 4° overnight with intermittent shaking, before washing three times in ice-cold calf serum and saline. Radioactivity in cell pellets was determined as shown. Similar results were obtained in three separate experiments. (*b*) Using the specific activity of the trace-labeled antigen arrays, we calculated the number specifically bound by comparing amounts bound in the absence and presence of excess inhibitor (*closed symbols*). In two independent experiments, FL-TCR$^+$ cells were cultured at 3×10^5 cells/ml with 3 ng/ml PMA and indicated doses of $FL_{960}Dex_{2300}$ (*open symbols*). Culture supernatants were harvested after overnight culture, and IL-2 production was assayed by determining proliferation of IL-2–dependent cells. For comparison, the IL-2 production in the one experiment (□) were normalized to values of the other at ~32,000 cpm; the actual maximum (*IL-2*) of the former experiment was ~80,000 cpm. (*c*) Linearity of IL-2 bioassay over required concentration range. Various dilutions of exogenous IL-2 were added to IL-2–dependent cells, whose proliferation was assessed by [^{3}H]thymidine incorporation after overnight culture.

the linearity of the IL-2 bioassay over the concentration range tested in Fig. 6 *b*. Such linearity indicates that the plateau and decrease in IL-2 concentrations induced at high TCR ligand concentrations, as shown in Figs. 1, 3, 4, and 6 *b*, is not due to saturation of the bioassay, which is linear over the relevant range.

The number of arrays specifically bound per cell at concentrations where IL-2 production is half-maximal is on the order of 100, as shown in Fig. 6 *b*. This number averages the heterogeneity in binding by FL-TCR transfectants (measured by FACS® analysis (Becton Dickinson & Co., Mountain View, CA); 21). Additionally, only a fraction of the cells may produce IL-2, even after repeated cloning and corrections for position in the cell cycle (36). We do not anticipate in this case that all 960 fluoresceins per array are simultaneously ligated; it is not yet possible to determine how many fluorescein ligands on arrays are actually bound to individual TCRs. For this reason, it is not yet possible to state exactly how many TCRs must be crosslinked by an array for minimal cell activation to occur. We can only measure how many ligands are required on the array to trigger activation.

Specific binding of arrays continues to increase at doses where IL-2 induction begins to fall, presumably because fewer and fewer ligands per array are occupying TCRs even as total array binding increases. This result further substantiates the notion that T cell activation does not always increase monotonically, and may be inhibited, with increasing TCR ligand concentrations and total TCR occupancy (30, 38).

Discussion

In this report, we have shown that T cell inhibition or activation can be controlled directly by antigen valence and array size, using soluble, physically defined antigen arrays and T cells transfected with TCR α and β chains previously shown to bind fluorescein. Large, multivalent antigen arrays trigger IL-2 production by FL-TCR$^+$ Jurkat cells in the presence of phorbol esters, but without accessory or MHC proteins, and they trigger calcium fluxes even in the absence of phorbol esters. In contrast, TCR occupancy by smaller, less multivalent antigen arrays is not sufficient for TCR-mediated signaling (as measured by calcium flux and IL-2 production), even though these arrays can inhibit activation by larger ones. Activation is specific, as IL-2 is not produced by untransfected cells in response to fluorescein polymers (Fig. 3) or by transfected cells in response to arrays of antigen analogs (21). Both activation and inhibition of transfected T cells occur with different carrier chemistries, indicating that the observed effects do not result from special properties of a particular class of carrier molecules. A model depicting the results is shown in Fig. 7.

Single cell calcium measurements (Fig. 5) demonstrate that occupancy of TCRs by smaller, oligovalent antigen arrays is not sufficient for induction of calcium fluxes. Rather, TCRs must be coordinately ligated by adequately large and multimeric antigen arrays to activate cells. Smaller, oligovalent arrays could therefore be used to antagonize T cell activation, presumably by competitively and nonproductively binding TCR. The results also verify that cell–cell contacts are not required for activation or inhibition by these soluble antigen arrays, as measurements were obtained by flow cytometry using dilute cell suspensions.

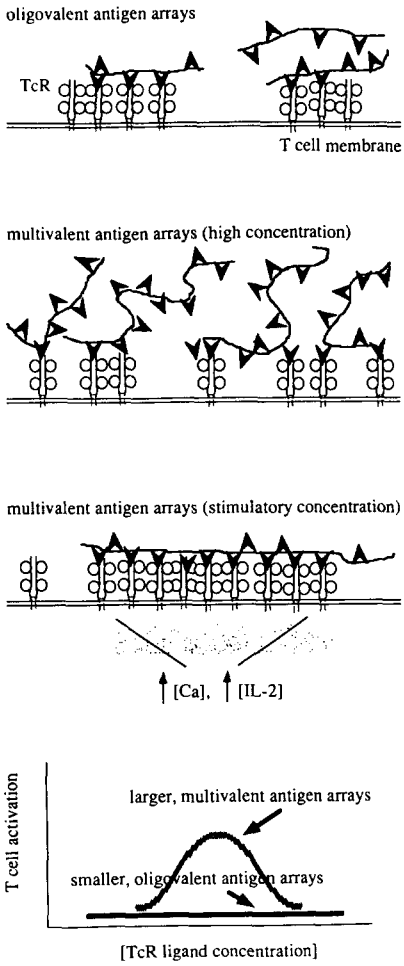

Figure 7. A model of T cell activation and inhibition by defined, soluble antigen arrays.

Both the inhibition or activation by arrays of different valence, and bell-shaped dose–response curves described here (Fig. 1, 3, 4, and 6) and elsewhere (30, 38), are consistent with a threshold number of locally occupied TCRs required for calcium fluxes or IL-2 induction to occur. According to this model, bell-shaped dose–response curves would result from multivalent, soluble ligands at high concentrations competing for neighboring receptors, minimizing receptor multimerization by individual arrays, and thereby reducing activation despite high overall receptor occupancy. TCR crosslinking by multivalent antigens at intermediate concentrations may result in enormously increased affinities of cytoplasmic ligands for intracellular sites on the multimerized transmembrane proteins, ultimately resulting in cell activation (39, 40). The precise molecular details of how tyrosine kinases, calcium fluxes, etc., are triggered by receptor mul-

timerization have not yet been elucidated. It is interesting that other cell types exhibiting similar bell-shaped dose–response curves may share similar receptor-associated signaling molecules (41, 42).

Oligovalent antigen arrays failed to stimulate, and in fact inhibited, T cell activation as seen in Figs. 3, 4, and 5. We infer that these smaller antigen arrays competitively and nonproductively bind TCRs. De Magistris et al. (43) recently reported that peptide-specific T cells could be inhibited by peptide analogs that bind equivalently to presenting MHC molecules. This was explained as competitive inhibition by the analogs in binding TCRs, but it is not yet clear why peptide–MHC–TCR complexes activate whereas peptide analog–MHC–TCR complexes do not. It is possible that peptide analogs could bind TCRs with lower affinities than nominal peptides, leading to insufficient TcR multimerization, but the precise relationship between ligation by antigen–MHC complexes on APCs, TCR multimerization, and T cell activation or inhibition remains unknown (44). It would be interesting to study systematically varied, soluble arrays of homogeneous peptide–MHC complexes, to correlate direct binding by these more "physiologic" TCR ligands with cell functions.

Using soluble peptide–MHC complexes to compete with antiidiotypic antibody in binding TCR, Matsui et al. (17) demonstrated that the K_d for TCR–MHC–peptide complex interactions is weak, ~ 4–6×10^{-5} M. The binding of solubilized TCR to MHC–peptide complexes on APCs is also weak, with $K_d \sim 5 \times 10^{-6}$ M (18). These values are similar to those calculated for interactions between FL-TCRs expressed by human T cell clones or bacterially expressed, single-chain TCRs and fluorescein, i.e., $\sim 5 \times 10^{-6}$ M and $\sim 5 \times 10^{-5}$ M, respectively (15, 16). In addition, a solubilized allogeneic MHC molecule antagonizes alloreactivity (presumably by competition with TCR) in vitro at 10^{-7} M, although this result is more difficult to interpret given the heterogeneity of MHC–antigen complexes (45). All of the affinities determined to date indicate that monovalent TCR-antigen binding is weak. However, multivalent or even oligovalent ligand–receptor interactions are energetically favored over monovalent interactions. Thus the effective affinity of arrays even of only a few TCR ligands could be much stronger than the weak affinities of monovalent TCR ligands (46). Peak cellular activation occurs at about 10^{-8}–10^{-7} M fluorescein when stimulatory arrays are used (Figs. 1, 3, 4, and 6), about three or four orders of magnitude less than the K_d values for monovalent fluorescein–TCR interactions noted above. We infer that the threshold number of ligands per array required for T cell activation may vary between antigens, and may be less for TCR ligands with greater affinities for TCRs, or for arrays with greater physical flexibility, ligand density, or steric accessibility. It is notable that the required number of ligands may be greater than the number of TCRs that must actually be crosslinked to trigger activation, if some of the ligands remain unbound. Thus our data demonstrate that at least two, and up to 50, local TCRs must be crosslinked by antigen to trigger activation, even though arrays with 20 fluoresceins per array did not activate the T cells.

A threshold in transcriptional activation may result from variable levels of transcription factor NF-AT, induced in activated Jurkat cells (36). It is possible that the threshold number of fluoresceins per array (>50), or the threshold number of arrays per cell, both required for IL-2 production by FL-TCR$^+$ T cells, may be linked to a threshold concentration of NF-AT or other factors required for transcriptional activation. We are currently attempting to determine whether inhibitory TCR antigen arrays induce negative regulatory signals that prevent calcium fluxes and lymphokine production, or whether such arrays (which occupy TCRs) induce insufficient or no intracellular signals. A candidate negative regulatory signal may be a nuclear transcription factor such as Nil-2-a, which negatively regulates IL-2 gene transcription in Jurkat cells (47).

Similarities in amino acid sequences of Ig and TCR framework domains, and in their V region gene structures, suggest that ligand–receptor interactions may be physically similar for both receptors (48). Several crystallographic structures of antibody–antigen complexes indicate small but significant, or even major, conformational changes in antibody Fab domains upon binding (49, 50), but such studies have not indicated conformational changes in hinge or Fc domains (51). Other workers have inferred that intermolecular interactions between coordinately ligated antigen receptors or associated signaling molecules could transduce signals across B cell membranes (42, 52–54). Thus, the structural studies to date suggest that some mechanism other than intramolecular conformational changes in Ig could account for transmembrane signaling by occupied surface receptors of B cells. By analogy (given similarities in primary sequences of Ig and TCR), there may be a requirement for multimeric receptor ligation in T cell activation, as postulated for B cells. Imminent crystallographic studies may help clarify this issue.

A unifying theme is emerging on the signaling properties of several transmembrane receptor molecules or complexes, including TCR, Ig, FcϵR, FcγR, epidermal growth factor receptor, platelet-derived growth factor, and insulin receptor. All have tyrosine kinase activities physically associated, and all require a threshold extent of receptor multimerization for intracellular signaling (55). These receptors may be distinguished from transmembrane receptors such as β-adrenergic receptors which putatively undergo intramolecular, transmembrane conformational changes upon binding monovalent ligands (52, 53). We infer that oligovalent ligands for the first class of receptors (i.e., antigens for TCR, Ig, FcR, etc.) can bind such receptors without cellular activation, and can inhibit activation by larger, multivalent ligand arrays. We are currently exploring pharmacologic uses of such oligovalent ligands as specific, competitive inhibitors.

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