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Effect of T-cell receptor antagonism on interaction between T cells and antigen-presenting cells and on T-cell signaling events

(T-cell activation)

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ABSTRACT **T-cell receptor (TCR) antagonism induced by complexes of antigen analogue with major histocompatibility complex (MHC) molecules results in efficient inhibition of antigen-dependent T-cell responses. We have investigated some of the possible mechanisms by which TCR antagonists bound to the MHC molecules of antigen-presenting cells (APCs) can inhibit T-cell activation. Using a nonstimulatory analogue of the antigenic peptide influenza hemagglutinin-(307–319), we showed that MHC/antagonist complexes completely inhibit very early intracellular events of antigen-dependent T-cell activation, such as inositol phosphate turnover and Ca^{2+} influx. In a parallel series of experiments, the effect of TCR antagonist peptide on membrane-related activation events was also investigated. It was found that MHC/antagonist complexes on the surface of APCs did not induce stable conjugates with T cells and, most interestingly, did not inhibit antigen-induced conjugate formation. Thus, our data suggest that antagonistic peptides do not interfere with the cellular events that are required for stable T-cell/APC conjugate formation but do inhibit early biochemical events required for T-cell proliferation. The data are discussed with respect to the role of surface receptor clustering in TCR antagonism.**

Highly effective T-cell receptor (TCR) competitive antagonists have been defined by using peptide analogues of the antigenic peptide influenza hemagglutinin (HA)-(307–319) presented in the context of the human class II major histocompatibility complex (MHC) molecule DR1 (1) on the surface of antigen-presenting cells (APCs). A DR1-restricted HA-(307–319)-specific T-cell clone was inhibited far more efficiently by the HA-(307–319) analogues (1000-fold) than by peptides capable only of inhibition by competition for MHC binding. This observation suggested that TCR antagonism may serve as a powerful approach to antigen-specific immunomodulation. Subsequent studies using different antigenic and antagonistic peptides suggested that peptide affinity for the TCR might play an important role in the inhibitory function of antigen analogues (2). However, the molecular mechanism involved in the phenomenon of TCR antagonism has remained elusive.

We have investigated the effects of TCR antagonism on early biochemical T-cell activation events such as inositol phosphate (IP) turnover and Ca^{2+} influx, as well as membrane phenomena such as T-cell/APC conjugate formation. We found that normal T-cell/APC coupling occurred whereas early intracellular biochemical events associated with T-cell activation were inhibited.

MATERIALS AND METHODS

Peptide Synthesis and Nomenclature. Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer and purified as described (3). Routinely, purity was >95% after high-pressure liquid chromatography. Analogues of HA-(307–319) containing single amino acid substitutions are named according to the substitution and its position; e.g., HA-Q313 has glutamine at position 313.

T-Cell Cultures and Proliferation Assays. The DR1-restricted, HA-(307–319)-specific T-cell clone Cl-1 was obtained as described (4). The DR1-homozygous, Epstein–Barr virus-transformed B-cell line LG-2 was used as APCs, either live or fixed in 0.5% paraformaldehyde for 20 min. LG2 cells (2.7×10^5 per ml in RPMI 1640 with 1% fetal bovine serum) were pulsed with antigen for 2 hr at 37°C and then washed in RPMI 1640/1% fetal bovine serum to remove unbound antigen. In prepulse assays, LG-2 cells were pulsed with a suboptimal dose of HA-(307–319), washed, incubated with various amounts of antagonistic peptides for an additional 2 hr, and washed again to remove unbound antagonist. Pulsed LG-2 APCs (4×10^4 per well) were cocultured with resting Cl-1 T cells (2×10^4 per well) in round-bottom microtiter plates in RPMI 1640/5% human serum. Twenty-four hours later, [*methyl*-^{3}H]thymidine (ICN) was added [1 μCi (37 kBq) per well]. After an additional 24 hr, cells were harvested onto glass-fiber filters, and thymidine incorporation was measured by liquid scintillation counting on a 1205 Beta plate counter (LKB).

IP Turnover and Ca^{2+} Influx Assays. IP turnover assays with Cl-1 T cells ($2–4 \times 10^7$ per ml) were prepared as described (5). Duplicate aliquots of T cells (2×10^6) were stimulated with LG-2 APCs prepulsed with antigen and/or antagonist as indicated. For measurements of intracellular Ca^{2+} influx, Cl-1 T cells were labeled with 2 μM indo-1 acetoxymethyl ester in medium for 30 min at 37°C, washed, and resuspended at 1×10^6 per ml. The emission of intracellular indo-1, excited at 350 nm, was measured at 400 nm with an SLM 8000 spectrofluorimeter (Aminco). Cl-1 cells were monitored for 30 sec to obtain a baseline before pulsed LG-2 APCs (2×10^6 per sample) were added. Changes in indo-1 fluorescence were followed over 250 sec before addition of anti-CD3 monoclonal antibody (OKT3, 1 μg/ml). Two minutes later, rabbit-anti-mouse immunoglobulin (10 μg/ml) was added as a crosslinking reagent. Fluorescence increases were followed for another 60 sec before lysing the cells with 0.1% Nonidet P-40 to determine the total Ca^{2+} content of the sample.

Expression of Interleukin 2 Receptor (IL-2R). LG-2 APCs (4×10^4 per well) were pulsed as indicated and incubated with Cl-1 T cells (8×10^4 per well) for 16 hr. Cells were washed, suspended at 6×10^5 per ml in phosphate-buffered saline/0.5% bovine serum albumin/0.2% sodium azide, incubated with anti-IL-2R antibody (100 μl, 1:400, anti-Tac) for 30 min on ice, washed, incubated with fluorescein-conjugated goat

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Abbreviations: APC, antigen-presenting cell; HA, hemagglutinin; IL-2R, interleukin 2 receptor; IP, inositol phosphate; MHC, major histocompatibility complex; TCR, T-cell receptor.

anti-mouse immunoglobulin F(ab')$_2$ (1:100, Sigma), and analyzed on a FACScan flow cytometer (Becton Dickinson) using LYSIS II software. Results were expressed as mean fluorescence of a gated T-cell population.

Analysis of Conjugate Formation. Pulsed live LG-2 APCs (4×10^4 per well) and Cl-1 T cells were coincubated in round-bottom microtiter plates at a 1:1 ratio. After 30 min the cells were washed in phosphate-buffered saline and suspended at 2×10^6 cells per ml. Cells were pipetted vigorously and transferred to a hemacytometer chamber for counting. Any LG-2 cell that stably bound to at least one T cell was counted as a conjugate. In each experiment samples of at least 200 LG-2 cells were randomized, coded, and counted in duplicate, and the percentage of LG-2 cells in conjugates was calculated.

RESULTS

TCR Antagonist Peptides Inhibit IL-2R Expression. Previous data have shown that antagonistic peptides are potent inhibitors of T-cell proliferation. Expression of the IL-2R is regarded as intermediate between "late" activation events, such as proliferation or IL-2 release, and "early" events, such as Ca^{2+} influx (6, 7). To examine the effect of the antagonistic peptide HA-Q313 on IL-2R expression, the Epstein–Barr virus-transformed cell line LG-2 was used in a prepulse assay. This assay was developed to avoid competition between antigen and antagonist at the MHC level and thus permits measurement of TCR antagonism (1). A 10-fold excess of antagonist (1 μg/ml) over antigen (0.1 μg/ml) was sufficient to inhibit IL-2R expression by $\approx$75% (Fig. 1).

TCR Antagonism Is Associated with Inhibition of Ca^{2+} Influx and IP Formation. Increased intracellular Ca^{2+} flux represents a crucial step in T-cell activation (8, 9). Previous experiments had shown that while significant Ca^{2+} influx could be detected in Cl-1 T cells in response to HA-(307–319), at 1 μg/ml no Ca^{2+} influx was detectable in response to APCs pulsed with the HA-Q313 antagonist alone at up to 100 μg/ml (1). In the experiments described here, the effect of the antagonist on antigen-induced Ca^{2+} influx was examined. HA-Q313 induced a profound dose-dependent inhibition of antigen-induced Ca^{2+} influx (Fig. 2*A*). Thymidine incorporation was inhibited to a similar extent over the same dose range of antagonist (Fig. 2*B*).

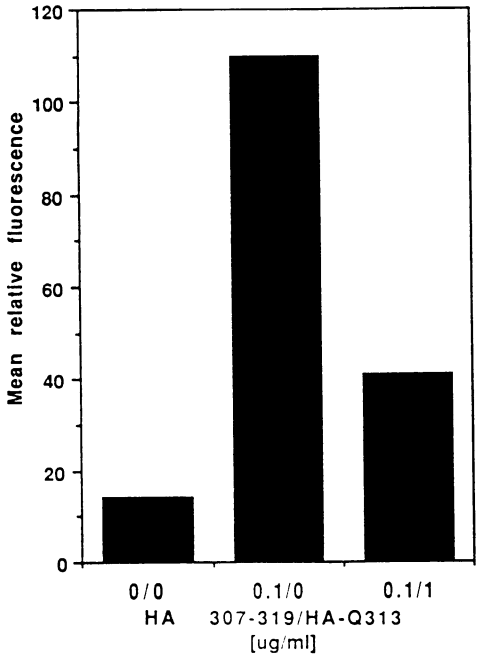

FIG. 1. TCR antagonist inhibits IL-2R expression. Cl-1 cells were incubated for 16 hr with fixed LG-2 APCs presenting the indicated doses of the antigen HA-(307–319) and the antagonist HA-Q313. Cl-1 cells were washed, and expression of IL-2R was measured by flow cytometry.

Generation of IP from inositolphospholipids precedes Ca^{2+} influx in the pathway of early signal transduction events in activated T cells (10, 11). In previous experiments Cl-1 cells did not respond to LG-2 cells pulsed with antagonist at up to 100 μg/ml whereas a dose-dependent increase of IP formation was observed with LG-2 cells pulsed with HA-(307–319) at 0.1–1 μg/ml (1). In the experiment shown in Fig. 3, IP formation induced by a suboptimal dose of antigen (0.2 μg/ml) was inhibited by about 70% with HA-Q313 antagonist at 10 μg/ml, and complete inhibition was reached with an antagonist dose of 100 μg/ml. Taken together, these results indicate that TCR antagonism is associated with complete inhibition of early intracellular events of T-cell activation.

Correlation Between Conjugate Formation and T-Cell Proliferation in an HA-Specific Response. Next, we investigated the effect of TCR antagonists on external membrane events that are associated with TCR–antigen–MHC interaction. In particular, conjugate formation between T cells and APCs is considered a prerequisite for T-cell activation and is dependent on presentation of a specific antigen to the T cell (12).

First, an antigen-dependent conjugate assay was established, according to a published method (13). Live LG-2 APCs were pulsed with antigen and incubated with Cl-1 T cells in a fashion similar to that used for proliferation assays. After 30 min of coculture, the cells were harvested, washed,

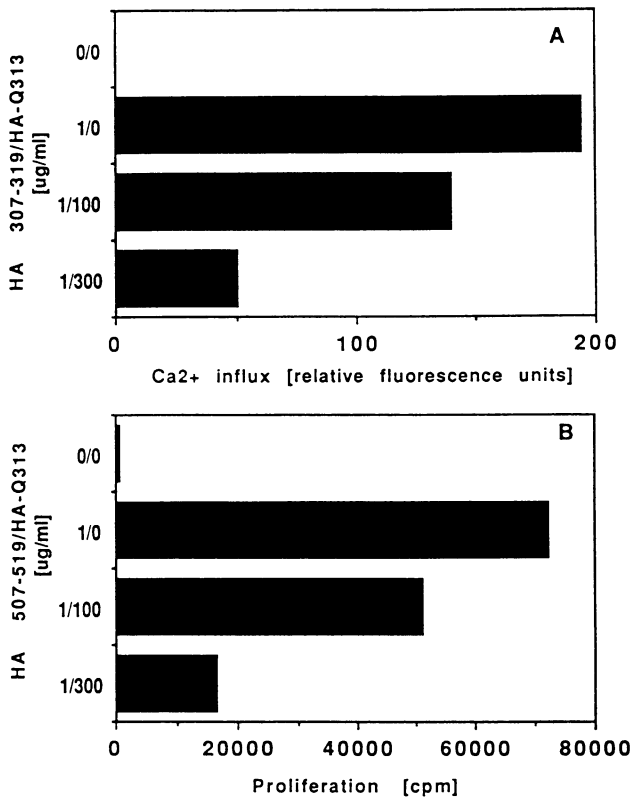

FIG. 2. TCR antagonism is associated with inhibition of Ca^{2+} influx. (*A*) Cl-1 T cells were labeled with indo-1 and then incubated with live LG-2 APCs prepulsed with the indicated doses of HA-(307–319) and HA-Q313. Changes in indo-1 fluorescence (400 nm) were monitored over time and net fluorescence after 250 sec was calculated. (*B*) For a functional control the same doses of antigen and antagonist were used in a proliferation assay. Cl-1 and fixed LG-2 cells were coincubated for 24 hr, [^{3}H]thymidine was added, and thymidine incorporation was measured after an additional 24 hr.

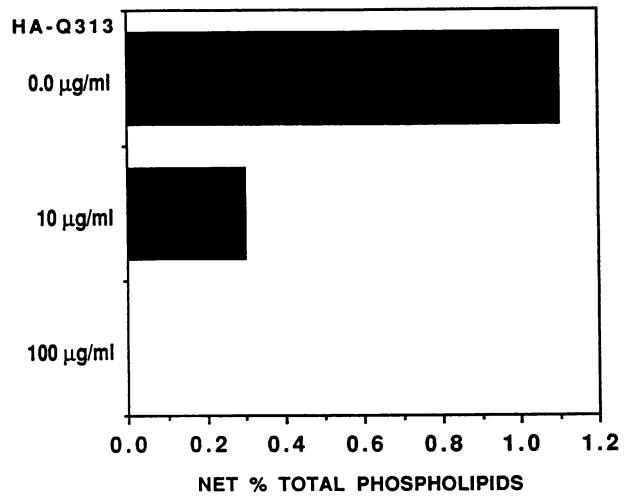

FIG. 3. TCR antagonism is associated with inhibition of phosphatidylinositol hydrolysis. LG-2 APCs were pulsed with HA-(307–319) (0.2 μg/ml), washed, and pulsed with the indicated doses of antagonist HA-Q313. Cl-1 cells were labeled with [^{3}H]inositol and coincubated with pulsed APCs. IP turnover was measured.

resuspended at 10^6 APCs per ml, and examined microscopically for conjugates. The background of nonspecific conjugates between unpulsed APCs and T cells was in the range of 2–8% in different experiments. Parallel cultures were incubated for 3 days so that thymidine incorporation could be studied as a functional control for the T-cell response associated with conjugate formation (Fig. 4). Conjugate formation, expressed as the percentage of LG-2 cells that bound one or more T cells, increased in an antigen dose-dependent manner. Conjugate formation above background was detected in response to as little as 10 ng of HA-(307–319) antigen per ml. A response of ≈20% specific conjugates was reached in response to antigen at ≥10 μg/ml. Significantly, the dose–response curve for conjugates closely paralleled the dose response for T-cell proliferation, thus supporting the immunological relevance of the established assay.

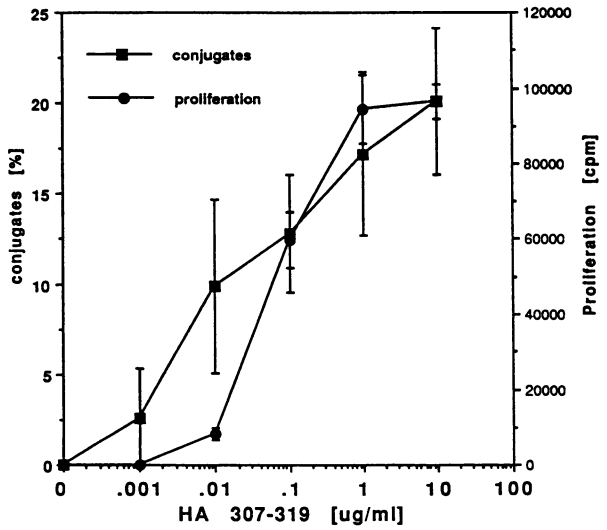

FIG. 4. Correlation between antigen-induced conjugate formation and proliferation. Live LG-2 APCs were pulsed with the indicated doses of HA-(307–313). Formation of conjugates was determined microscopically after 30 min of coincubation with Cl-1 T cells (■). In parallel, pulsed fixed LG-2 cells were coincubated with Cl-1 T cells for 24 hr, [^{3}H]thymidine was added, and thymidine incorporation was determined 24 hr later (●). The graph shows the mean ± SD of three independent experiments.

Effect of Antagonist Peptides on the Formation of Conjugates. To investigate whether APCs pulsed with antagonist peptides could also induce stable conjugates with T cells, live APCs were pulsed for 2 hr with a single high dose (100 μg/ml) of various antagonists and incubated with Cl-1 cells for 30 min. None of the antagonists tested induced the formation of a significant percentage of conjugates (Fig. 5).

Next, the induction of specific conjugates in response to APCs prepulsed with a suboptimal antigen dose and subsequently pulsed with graded doses of the antagonistic peptide HA-Q313 was measured (Fig. 6). While T-cell proliferation was inhibited with as little as 1 μg of antagonist per ml, no inhibition of conjugate formation was detected over the entire dose range tested. Thus, it was concluded that in contrast to their inhibitory effects on IP turnover and subsequent intracellular biochemical events, TCR antagonists did not interfere with the initial cellular interaction events in the T-cell activation pathway.

Effect of Antagonist Presented on Different APCs Than Antigen. In the next series of experiments, antigenic and antagonistic peptides were presented either on the surface of the same APC (prepulse) or on the surface of separate APCs (separate pulse). T cells were coincubated with two populations of LG-2 APCs, one pulsed with a suboptimal amount of antigen (0.05 μg/ml) and the other pulsed with a graded dose of the antagonist (0.001–100 μg/ml). In parallel, an assay was performed with the standard prepulse protocol. As expected, proliferation of T cells incubated with APCs presenting antigen and antagonist (prepulse) was readily inhibited (Fig. 7; IC_{50} = 1 μg/ml). In contrast, no inhibition of Cl-1 proliferation was observed when up to 100 μg of antagonist per ml was presented by different APCs. These results indicate that TCR antagonism cannot be explained by simple competition of MHC/antigen and MHC/antagonist for TCR binding.

DISCUSSION

A previous report (1) demonstrated how simple analogues of antigenic peptides can effectively inhibit antigen-specific T-cell responses when presented in the context of MHC molecules on APCs. These inhibitory antigen analogues did not induce activation signals in T cells, nor could their effect be attributed to MHC blockade or induction of tolerance (1).

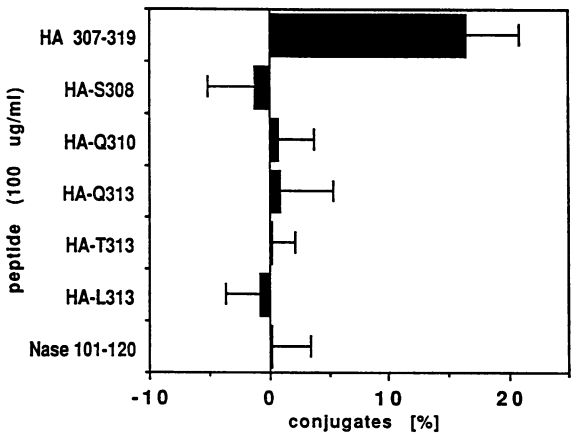

FIG. 5. Conjugate formation by various antagonists. Live LG-2 cells were pulsed with HA-(307–319) or antagonistic analogues at 100 μg/ml for 2 hr. The staphylococcal nuclease-(101–120) peptide (Nase 101-120) is an unrelated DR1-binding peptide and was used, also at 100 μg/ml, as a negative control. Washed LG-2 cells were then coincubated with an equal number of Cl-1 T cells. After 30 min the cells were counted under a microscope and the percentage of LG-2/Cl-1 conjugates was calculated. The graph represents the mean ± SD of three independent experiments.

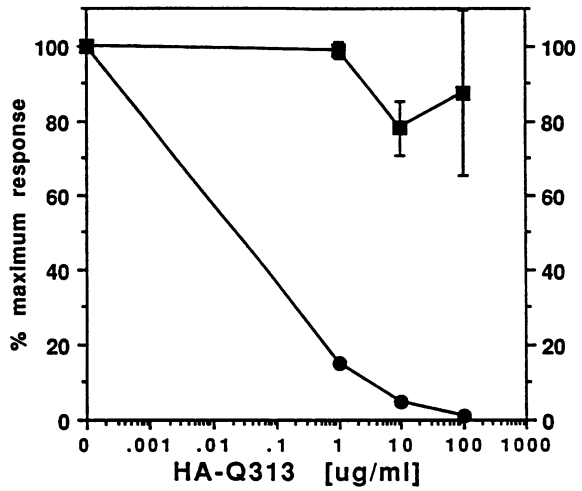

FIG. 6. Effect of TCR antagonist on T-cell conjugate formation and proliferation. LG-2 cells were first pulsed with a suboptimal dose of the antigen HA-(307–313) (0.05 μg/ml) for 2 hr, washed, and subsequently pulsed for 2 hr with graded doses of the antagonist peptide HA-Q313. Prepulsed LG-2 cells were coincubated with Cl-1 cells for 30 min. The percentage of conjugates containing one LG-2 cell and one or more Cl-1 cells was determined under a microscope (■). The same pulses of antigen and antagonist for LG-2 were used in a Cl-1 proliferation assay (●). Data are plotted as a percentage of the maximal response to compare the two different assays.

It was therefore concluded that they acted as antagonists of the TCR.

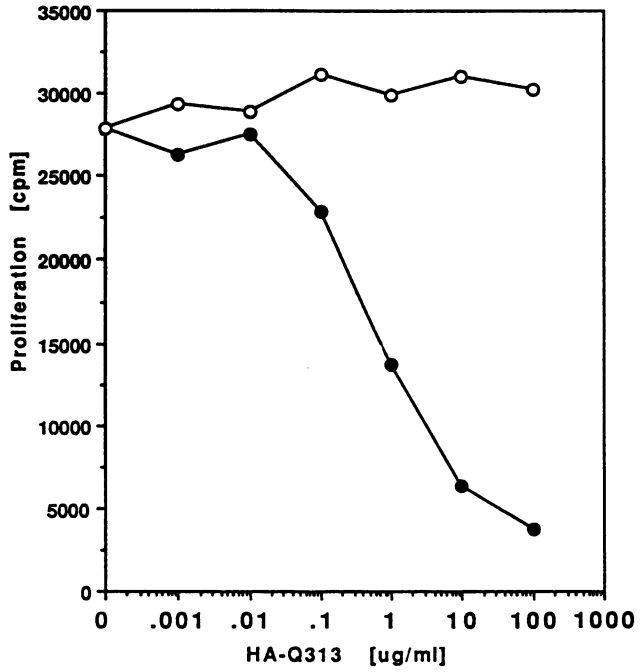

FIG. 7. Effect of the antagonist presented on different APCs than antigen. For the separate-pulse experiment (○), two differently pulsed populations of fixed LG-2 APCs were used: one population was pulsed with a suboptimal dose of the antigen HA-(307–319) (0.05 μg/ml); the other population was pulsed with the indicated dose of the antagonist HA-Q313. Equal numbers of the two populations (10^5 per ml each) were incubated with Cl-1 T cells (2×10^5 per ml) in microtiter plates. In the prepulse experiment (●), one population of LG-2 cells was first pulsed with a suboptimal dose of antigen (0.05 μg/ml) for 2 hr, washed, and then pulsed with the indicated doses of the antagonist for another 2 hr. Prepulsed LG-2 cells (10^5 per ml) and equal amounts of unpulsed LG-2 cells were used for coincubation with Cl-1 T cells (2×10^5 per ml).

In the present study, we further investigated the mechanism of TCR antagonism. Using the HA-(307–319)-specific, DR1-restricted prepulse assay, previously described (1), we found that later events in the T-cell activation cascade, such as IL-2R expression, as well as immediate intracellular steps, such as increases of cytoplasmic Ca^{2+} or IP formation (14), were inhibited by antagonistic peptides. By measuring T-cell/APC conjugates, we found that the dose responses of conjugate formation correlated very well with the antigen dose-dependent proliferative response. To our surprise, this antigen-dependent conjugate formation was not inhibited at all by TCR antagonists. This finding, together with the observation that antagonist presented by a different APC than the one presenting antigen was noninhibitory, strongly suggested that the mechanism of antagonism is more complicated than simple competition for TCR binding.

The differential effects of the antagonist on conjugate formation on the one hand and Ca^{2+} flux and IP formation on the other imply a multistep T-cell activation pathway: The molecular mechanism most likely necessary for stable conjugate formation functions normally in the presence of the antagonist, whereas IP turnover and subsequent signaling events leading to lymphokine secretion and a proliferative response are inhibited. Interestingly, in this context O'Rourke and Mescher (15) have recently demonstrated that CD8-mediated adhesion of cytotoxic T lymphocytes can be achieved upon recognition by signals distinct from those involved in IP turnover and activation. There, antigen binding to the TCR would result in "horizontal" signaling transferred to the membrane co-receptors first, leading to CD8-related adhesion events, followed by "vertical" signaling into the cell resulting in a T-cell response such as phosphatidylinositol hydrolysis. Inasmuch as cytotoxic T cells and T helper cells share the major mechanisms of immediate and early intracellular signaling (16), this cascade of activation events could also apply for T helper cells. Both CD8 on cytotoxic T cells and CD4 on T helper cells are coupled to the $p56^{lck}$ protein-tyrosine kinase (17), which is likely to be involved in the activation of phospholipase C leading to phosphatidylinositol hydrolysis (18) and is a likely candidate to explain the protein-tyrosine-kinase dependence of adhesion (15). In our experiments, the antagonist apparently did not interfere with the initial membrane-associated horizontal signaling, involving increased affinity of adhesion molecules on Cl-1 cells. Stable formation of conjugates could therefore be induced, while the subsequent vertical signaling into the cell was effectively blocked by the antagonist. Another example showing that the TCR has the capacity of differential signaling was described (19), when a single amino acid substitution in the antigen resulted in a partial agonist which could induce cytokine (interleukin 4) production in T helper cells but could not induce a full proliferative response.

Effective signal transduction in T cells involves engagement of the TCR/CD3 complex in a mutual interaction with MHC/antigen (20). The CD4 molecule on the T-cell surface was proposed as a co-receptor (21), syn-capping with TCR/CD3 clusters (13, 22), and enhancing T-cell activation (23). We therefore examined whether MHC/antagonist complexes might interfere with clustering events on the cell surface. In our experience, however, it was impossible to correlate antigen dose with the amount of capping (data not shown). We observed that suboptimal to optimal antigen doses (0.1–1 μg/ml) did not induce detectable capping of TCR and CD4 in antigen-specific conjugates. An antigen dose 10- to 100-fold higher than that required for proliferative responses had to be used to induce appreciable levels of capping, which then was not inhibitable by antagonist. This lack of correlation between capping and proliferation implies that the massive aggregation required to visualize capping is not relevant to signaling, but rather, as in other systems (24), nonvisible

microclustering at lower antigen concentrations represents the relevant aggregate. If the clustering of TCRs into dimers or oligomers is a critical event in the signaling of a T cell, how then do antagonist peptides function? Two mechanisms can be envisioned on the basis of our recent observation that antagonist peptides probably have a lower affinity for the TCR than the antigen (2). First, the low-affinity interaction between antagonists and TCRs could, because of a rapid off-rate, prevent critical clustering of the receptors required for signaling. Alternatively, it could be speculated that the multiplicity of steps involved in T-cell signaling reflect conformational changes in the TCR induced by the bound peptide/MHC complexes (25, 26). It was suggested previously that both crosslinking and conformational changes in the TCR upon antigen recognition may be required for optimal T-cell activation (27). If the low-affinity interaction of the TCR with MHC/antagonist is not sufficient for the induction of this TCR conformational change, then formation of mixed clusters in the T-cell binding site induced by an APC presenting both antigen and antagonist could lead to blockade of intracellular signaling and inhibition of T-cell proliferation. Recently, a similar mechanism of signal inhibition by nonfunctional hetero complexes has been demonstrated for the p53 molecule (28, 29).

In this light, we hope that TCR antagonists might prove to be a useful tool to gain further insight into the membrane-associated molecular events of signal transduction required for T-cell activation.

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