Major Histocompatibility Complex Independent
T Cell Receptor-Antigen Interaction: Functional
Analysis Using Fluorescein Derivatives

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Summary
We have isolated T cell receptor (TCR) cDNAs from fluorescein (FL)-specific human T cell
clones (unF1.), and transferred them to TCR a/FL Jurkat cells in order to study direct FL-binding
to the TCR. Using either FL-conjugated polymers (FIrpolymer) or FL-substituted Sepharose
beads, we are able to demonstrate the direct binding of antigen to the T cell surface, and the
functional activation of the T cell transfectants. We present evidence against the involvement
of major histocompatibility complex (MHC) molecules or antigen presentation in the interaction
of FL with the a/FL TCR transfectants. Additionally, we have examined the effect of ring
substitutions on the FL molecule as well as specific alterations of substituents attached to the
5' position, and we have found that all of them interfere with the functional recognition of
the a/FL TCR. These experiments demonstrate that TCRs like antibodies have intrinsic
affinities for antigen, even without the involvement of MHC molecules.

Molecular (reviewed in references 1 and 2) and cellular
(3–7) studies have identified the TCR for antigen as
a 90 kD a/β heterodimer on the T cell surface. The primary
function of TCRs is in the recognition of antigen in a com-
plex process that involves gene products of the MHC on APC
(8, 9). An important function of these MHC molecules is
to bind and present peptide fragments of protein antigens
for recognition by TCR. Defined peptides which represent
appropriate epitopes in foreign antigens will selectively bind
particular MHC molecules (10, 11). These peptides, when
presented by APC, will activate T cell clones (12) or trans-
flectants expressing TCRs of those clones in an antigen specific
and MHC-restricted fashion (13, 14). MHC molecules also
interact with CD4 (class II MHC) or CD8 (class I MHC)
cell-surface receptors on T cells in a manner which promotes
adhesion of the interacting cells and signal transduction
(15–18). Transfection of antigen specific and MHC-restricted
TCRs into host cells of irrelevant specificity results in the
transfer of specificity for both MHC and antigen (19–21).
These results demonstrate that the TCR is the only mole-
cule necessary to establish antigen specificity and to confer
MHC restriction on a T cell.

There are few reports which demonstrate that antigen can
interact with TCR in a manner that is independent of MHC
(22–25). A novel series of murine (26) and human (27) T
cells which recognize the hapten fluorescein (FL) were
generated to determine if the TCR has the intrinsic capacity
to bind antigen independent of MHC and accessory cells.
These clones were shown to interact with polymers substituted
with multiple FL molecules independently of accessory cells.
Several of the human FL-specific T cell clones can be acti-
vated to secrete IL-2 by incubation with Sepharose beads
conjugated with FL-isothiocyanate (FITC; reference 27).
Significantly, solubilized forms of the surface TCR from these
clones specifically bound to FITC-substituted Sepharose
beads in affinity chromatography experiments (27). However,
whether antigen recognition and functional cellular adhe-
sion of these clones could occur in an absolutely MHC inde-
pendent fashion was unclear. To address this issue, a transfection
model using a functional T cell tumor with the TCRs from
the FL-specific T cell clones was developed to study the di-
rect binding of antigen to the TCR in the absence of APC

Abbreviation used in this paper: FL, fluorescein.
and accessory molecules. We present the results of one such cellular transfection system which demonstrate that in this system the TCR can engage the hapten FL under defined circumstances without MHC interaction. In addition, we have been able to define several parameters of the TCR's ability to recognize the FL molecule by examining the effect of chemical substitution on the FL ring system in a direct binding and activation assay with transfected cells. This system may be useful in the eventual determination of the precise antigen binding site of a TCR.

Materials and Methods

Cell Lines and FL Mucins. The cell lines shown in Table 2 were propagated in RPMI-1640 (JRH Biosciences, Lenexa, KS) with the standard additions which include glutamine, Pen-Strep, and 10% FCS (JRH Biosciences). Transfectants were routinely grown in RPMI-1640 (JR Scientific) medium containing Hygromycin B (Sigma Chemical Co., St. Louis, MO) at 0.5 mg/mL and/or G418 (Gibco Laboratories, Grand Island, NY) at 0.5 mg/mL. CTLL-2 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI-1640 as described above with the added supplementation of 50 uM 2-ME and 10 u/mL rIL2 (Cetus Corp., Emeryville, CA).

The properties of the FL-Mucins used in this publication are summarized in Table 3 below. The preparation of FL-conjugated polyacrylamide, dextran, or Ficol has been previously described in detail (26, 28, 29).

cDNA Cloning. T cell clones RFL 3.8 and 3.51 were stimulated by FITC as previously described (27), and grown to a density of 10^6 cells/mL. Poly A+ RNA was obtained by standard methods after 2x binding to Oligo DT Cellulose (T7, Collaborative Research, Waltham, MA). 5 ug of the poly A+ RNA was used in the first strand synthesis reaction, which was immediately followed by the second strand synthesis protocol of Gubler and Hoffman (30). The double stranded cDNA was blunt ended by T4 DNA Polymerase, Eco RI linkers were attached after methylation by Eco RI methylase, and the cDNA was size fractionated according to standard methods (31). 0.2 ug of cDNA was ligated to 1 ug Eco RI cleaved and purified A+gt-10 arms (72) overnight at 12°C. Half of the ligated DNA was packaged into lambda phage packaging extract (Stratagene™, La Jolla, CA) for 2 h at room temperature, and it was inactivated by the addition of 0.5 mL TM (10 mM Tris-HCI, pH 7.5 and 10 mM MgCl2) and 10 uL chloroform. The phage was titrated on both permissive (HFL) and restrictive (C600) strains

Electroporation was carried out using a modification of a procedure developed for human PBL and T cells (39). Briefly, cells were split the night before transfection to a density of 0.3–0.4 × 10^6/mL. The following day cells were centrifuged at 2 × 10^7 cells/mL in RPMI-1640 containing 10% FCS (complete medium), and 0.4 mL were suspended in an electroporation cuvette together with plasmid DNA at 100 µg/mL. The suspended cells were incubated on ice for 10', and then 1 pulse of electroporation was carried out at 200–250 V and 800–960 Farad (BRL Cell-Porator). The shocked cells were left on ice for an additional 10', then pipetted and washed in complete medium. After 2 d of recovery, the living cells were separated by centrifugation through Ficoll and plated in 96-well plates in Hygromycin B (0.5 mg/mL) or Hygromycin B + C418 (0.4 mg/mL + 0.5 mg/mL) to select for transfecants. Typically, we found a frequency of 1 in 10^4 transfecants using this procedure. Positive clones were identified initially by growth in selection medium, and then expanded for FACS and Northern analysis.

FACS Analysis. We examined 50 clones containing either the RFL3.8 or RFL.3.51 β chain by FACS using a mAB against CD3 (RW2-8C8; reference 40) and staining with a FITC conjugated goat anti-mouse secondary antibody (Cappel Laboratories, Durham, NC). The cell staining procedure was according to Siliciano et al. (27), and propidium iodide (PI, 1 µg/mL was added to enable exclusion of dead cells. Cells were analyzed on a Becton-Dickinson FACSV equipped with dual lasers (argon and UV). Both 90° light scatter and the fluorescence channel (510 nM) were used to gate the single cell population that was free of PI. The representative plots shown in Fig. 2 were obtained from analysis of 2 × 10^4 cells. Fluorescent microspheres were used to correct changes in amplitude in the fluorescence channel when comparing samples analyzed on different days. In general, the expression of the RFL3.8...
\(\beta\) chain clones is 4-5 x greater than from the FL3.51 \(\beta\) chain in combination with the endogenous Jurkat \(\alpha\) chain (unpublished data). However, Northern analysis of a series of RNA samples from the FACS analyzed clones did not reveal any significant difference in the RNA expression (unpublished data). Others have found a difference in TCR pairing ability between chains when the RNA levels were similar (41).

In the case of the FL-Polymers, 10\(^6\) cells to be analyzed were washed twice in WM (HBSS containing 2% newborn calf serum [JRH Biosciences], 10 mM Hepes (pH 7.4), 0.1% glucose, and Pen-Strep). The various FL-Polymers were added to the cells in 50 \(\mu\)L of the same buffer at a final concentration of 30 \(\mu\)g/mL. The cells were incubated in an ice bath for 30', then washed twice in WM, and finally resuspended in 0.5 mL of WM containing propidium iodide at 1 P.g/mL. The samples were kept ice cold until analysis, typically in <2 h, otherwise the background staining would rise dramatically. As with the antibody analysis, 2 \(\times\) 10\(^6\) cells were analyzed per profile.

Cell sorting was accomplished with the aid of a Cicero analysis system (Cytomation) on the FACS IV, and using standard techniques of cell preparation using azide-free medium. The brightest 5% of cells were collected, expanded, and resorted to obtain a relatively homogeneous population (see Fig. 2).

**Cell Activation Assays.** 0.6 \(\times\) 10\(^6\) cells to be treated with agents (FL-Polymer, etc.) were washed twice to remove antibiotics, and resuspended in one well of a 24 well plate (Falcon) with 0.6 mL normal growth medium. The cells were incubated with the agents for 24 h, and the supernatants collected by centrifugation and stored at 4°C. FL-Polymers were added to 1 \(\mu\)g/mL with or without 1 or 10 ng/mL PMA. Sepharose beads in a volume of 20 \(\mu\)L (determined to be saturating by titration) were added to the cells to be stimulated for 24 h. FL-Cad was added as dilutions of a dimethylformamide stock solution of 100 mM.

CTLL-2 cells are cultured in 4U/mL recombinant IL-2 (Cetus Corp.), and every 3-4 days they are restimulated with fresh IL-2. The cells to be tested are washed four times, and resuspended in growth medium containing 50 \(\mu\)M mercaptoethanol. 10\(^6\) CTLL were cultured in 100 \(\mu\)L medium in 96-well plates (NUNC) with dilutions of IL-2 containing supernatants up to 50%, or a series of FL-2 dilutions, 0.5 or 5 ng/mL PMA, or medium without additions. Each supernatant was tested in triplicate, and each experiment was repeated twice or more. The data presented is an average of all the experimental determinations for each point, with no separate determination having a standard error of >20% from the mean.

**Preparation of Sepharose Beads Substituted with FL Derivatives.** We followed the techniques described in (27), except for the following modifications. 0.5 gram of CNBr Activated Sepharose 4B (Pharmacia) was washed according to the manufacturer, and reacted with a 100 x excess of 1,6 diaminohexane in 0.1 M NaHCO\(_3\) (pH 8.3) for 2 hours, then washed again 2 x with coupling buffer. Either a 1 or 8 mM solution of FITC or X-isothiocyanate was reacted with the beads in 0.1 M borate buffer for 60' at 37°C. Alternatively, when the FL derivative was in the amine form, it was directly reacted with the Sepharose beads without the previous spacer coupling. The ligand was washed away, and the beads were neutralized according to the manufacturer. Ethanolamine was reacted directly with the Sepharose beads, and they were neutralized. The beads were stored at 4°C under foil in PBS. Each batch of derivatized bead was adjusted in concentration to 2 \(\times\) 10\(^6\) beads/mL. All of the beads are greater in size than 50 \(\mu\)M. The FL derivatives containing primary amines were all obtained from Molecular Probes (Eugene, Oregon), while some of the isothiocyanate derivatives were also obtained from Sigma Chemical Co.

**Results**

**Cloning and Characterization of the \(\alpha_FL\beta_FL\) TCRs.** As a first step in understanding the structural interactions between FL and the TCR, we cloned the TCR \(\alpha\) and \(\beta\) chains from cDNA libraries constructed from two human T cell clones referred to as RFL 3.8 (CD4\(^+\)) and RFL 3.51 (CD8\(^+\), Fig. 1 A-F and reference 27). We made V-J segment probes from each of the 4 cDNAs, and hybridized them to various genomic DNAs (Fig. 1 A-D). Each of the 4 V-J segment probes hybridized to a cell-specific rearranged band in the appropriate DNA sample. For instance, the RFL 3.8 \(\alpha\) cDNA V-J segment hybridized to a 3.5 kb Eco RI and a 3.2 kb Hind III fragment in DNA from clone RFL 3.8 and a related CD4\(^+\) FL-specific T cell clone RFL 3.1 while DNAs from other human T cell and non-T cell lines had hybridizable bands at different positions (Fig. 1 A, compare lanes 1 and 2 versus 3-5 and lanes 6 and 7 versus lanes 8-10). Note, that Jurkat DNA has deleted the V segment that encodes the FL-specific V\(_\alpha\) gene segment (Fig. 1 A, lanes 4 and 9). In each case, when an FL-specific V-J probe was hybridized to various test DNAs, only the expected cell line from which the V-J segment was originally cloned demonstrated rearrangement.

The human gene segments used in each of the 4 cDNAs are shown in Table 1. The \(\alpha\) chain from RFL3.8 contains a V gene segment which is almost identical to V\(_3\)17 (2), while the J segment was identical to a previously identified J segment termed F in the recent nomenclature of (2). Additionally, the \(\beta\) chain from RFL 3.8 uses a V gene segment that is similar to other members of the V\(_3\)13.2 family, although there are significant (16/92) amino acid differences (2). In the case of clone RFL 3.51, the \(\alpha\) chain contains a V segment that is similar to the V\(_\alpha\)2 family, while the J segment (L) has been previously identified (2). The \(\beta\) chain from RFL 3.51 uses a new member of the V\(_\alpha\)2 gene family that we have designated 2.5. The sequence of each of the individual chains is shown in Figure 1F. There is a striking similarity

| Clone | Phenotype | Specificity | Chain | V | D | J | C |
|-------|-----------|-------------|-------|---|---|---|---|
| RFL 3.8 | CD4\(^+\)CD8\(^-\) | FL-Class II | \(\alpha\) | 17 | - | F | C\(_\alpha\) |
| RFL 3.51 | CD4\(^+\)CD8\(^-\) | FL-Class I | \(\alpha\) | 2.8 | - | L | C\(_\alpha\) |

**Summary of the gene segments used in the formation of the TCR cDNAs cloned from the FL-specific T cell clones shown on the left side of the table (27). The symbols V, D, J, and C are defined in the legend to Fig. 1. CD4 and CD8 expression were determined from both antibody (27) and Northern analysis (Fig. 1 E).**
TCR αFL CHAIN VARIABLE REGION SEQUENCES

-10 0 10 20 30

| 30% CONSERVED | 30% CONSERVED |
|---------------|---------------|
| RFL 3.8       | RFL 3.51      |

| 40 50 60 70 80 |
|----------------|
| RFL 3.8       |
| RFL 3.51      |

| 90 100 110 120 |
|----------------|
| RFL 3.8       |
| RFL 3.51      |

TCR βFL CHAIN VARIABLE REGION SEQUENCES

-10 0 10 20 30

| 30% CONSERVED | 30% CONSERVED |
|---------------|---------------|
| RFL 3.8       | RFL 3.51      |

| 40 50 60 70 80 |
|----------------|
| RFL 3.8       |
| RFL 3.51      |

| 90 100 110 120 |
|----------------|
| RFL 3.8       |
| RFL 3.51      |

EBV BASED EXPRESSION PLASMIDS WHICH ARE MAINTAINED AS EPISOMES IN HUMAN CELLS

p266.1/a
confers G-418®

p220.2/b
confers Hyg®

Diamond et al.
in that both TCRβ cDNAs use the same Jβ, and a region in both TCR α cDNAs between aa 67–74 shows similarity where most TCRs are divergent. Finally, Northern analysis further confirms the specificity of the V-J segments for the individual T cell clones, as well as the absence of minor species of contaminating TCR RNAs (Figure 1E).

**Transfection and FACS Analysis.** We subcloned both pairs of FL-specific TCR cDNAs into a novel series of expression vectors based upon the Epstein-Barr virus (EBV) (Figure 1G). The expression of the EBNA1 gene allows the OriP gene segment to function as an origin of replication, and as a result the plasmids are maintained as episomes in human cells (36). The vectors were introduced into the TCR β− mutant of Jurkat, 31-13 (42) by electroporation (39). Single colonies were selected and expanded in Hygromycin containing medium, and analyzed by fluorescence activated cell sorting (FACS) to determine whether the introduction of a β TCR cDNA had restored expression of surface CD3 expression (42, 43). Figure 2 shows that compared to the parental line Jurkat, 31-13 is negative for CD3. In contrast, the single transfectants 3C11 or 42 (Table 2) which express βFL TCR chains from clones RFL 3.8 and RFL 3.51 respectively, have almost completely restored surface CD3 expression. Subsequently, we introduced the vector p266.1/α (Figure 1G) carrying the αFL TCR chain of clone RFL 3.8 into the cell line 3C11 and administered the dual selection of G-418 and hygromycin. Surprisingly, we observed colonies within 1 week, at a frequency 100 fold higher than in the initial transfection with p220.1/β (Figure 1G). This may be the result of the already established EBV plasmid supplying EBNA1 in trans to the incoming plasmid which has its own OriP site (37).

In order to determine if both the αFL and βFL TCR chains were being expressed, individual drug-resistant clones were expanded, and subsamples were stained with the FITC containing polyacrylamide polymer, FL90PA2300 (see Table 3 for a description of the FL polymers and Table 2 for the cell transfectants) and analyzed by FACS (Fig. 2). Only cell lines such as A5A6 (Table 2) which contain both the α and β TCR chains from clone RFL 3.8 bound polymer, while the parental line and other transfectants which contained just a βFL chain were negative. The degree of binding of the FL-Polymer to the αFLβFL cell lines is reduced, because there are two types of TCR on the cell surface. One type is the functional

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**Table 2. Cell Line Properties**

| Cell line      | TCR chains          | Selection conditions | Reference          |
|----------------|---------------------|----------------------|--------------------|
| Jurkat (J77)   | α and βββ           | None                 | Alcover et al. 1988|
| 31-13          | αβββ                  | None                 | Alcover et al. 1988|
| 3C11N          | αβββ                 | Hygromycin           | This paper         |
| 43N            | αβββ                 | Hygromycin           | This paper         |
| A5A6           | αβββ + βββ           | Hygromycin + G-418   | This paper         |
| 1C5            | αβββ + βββ           | Hygromycin + G-418   | This paper         |
| E3E4           | αβββ + βββ           | Hygromycin + G-418   | This paper         |
| 42             | αβββ + βββ           | Hygromycin           | This paper         |
| E2             | αβββ + βββ           | Hygromycin + G-418   | This paper         |

Table 2 summarizes the properties of the cell lines used in this publication. Details of the derivation of the transfectant cell lines and their growth conditions can be found in Materials and Methods.
Techniques of analysis to determine average molecular weights of polymer preparations were done as previously described (26). FL content of polymers was measured as described in (27).

Figure 3. Proliferation assays using supernatants from T cell lines activated by FL-polymer reagents. (A) Antibody and Calcium Ionophore stimulate IL-2 production in T cells. The T cells indicated in the box were treated with the reagents indicated on the bottom of (A) for 24 h as described in Materials and Methods. 50% supernatants were incubated with CTLL-2 cells for a total of 24 h including a 6-h pulse with 3H-thymidine (1 μCi). The cells were harvested with the aid of a semi-automatic cell harvester apparatus (Skatron Inc., Sterling, VA). The bars represent data collected from at least two experiments with each treatment done in triplicate (n = 6) with an SE of <20%. The cell lines are described in Table 2. (B) FL-polymer treatment of T cell transfectants. The cell lines indicated in the box on the left of (B) were treated with the indicated reagents (bottom) as described in (A). The FL-polymers FLD (FL100PA2500), FLP (FL100PA3000), or FLF (FL45Fic220) are described in Table 3.

| Designation   | Polymer backbone | Average mol wt (x 10^-5) | Antigen valence (FL groups/molecule) | Epitope density (mol FL/g polymer x 10^4) |
|---------------|------------------|--------------------------|--------------------------------------|-----------------------------------------|
| FL70PA2300    | Polyacrylamide   | 23.0                     | 790                                  | 2.91                                    |
| FL100PA2500   | Polyacrylamide   | 25.0                     | 1,000                                | 2.50                                    |
| FL9PA3000     | Polyacrylamide   | 3.0                      | 95                                   | 3.16                                    |
| FL100Dex2500  | Dextran          | 25.0                     | 1,000                                | 2.50                                    |
| FL110Dex330   | Dextran          | 3.3                      | 110                                  | 3.00                                    |
| FL45Fic220    | Ficoll           | 2.2                      | 65                                   | 3.38                                    |

Table 3. Properties of FL-Polymers

αβ,βFL TCR, while the other is the mixed TCR containing αFL + β, which is non-functional as is the case in the cell line 3C11N (Fig. 2). Our analysis of the TCRs from clone RFL 3.51 has been hampered, because the β chain is unstably expressed in transfected 31-13 cells, so we are unable to test whether the αβ heterodimer from RFL 3.51 binds FL. It is clear in the cell lines we tested that the α or β TCR cDNA from RFL 3.8 alone or in combination with other TCR chains does not confer FL binding (Fig. 2, compare clones 3C11 or E2 with A5A6). Therefore, for clone RFL 3.8, we assume that both the α and β TCR chains contribute to antigen binding.

Stimulation of IL-2 Secretion by FL. As reported previously, FL-Polymer binding to the T cell clone RFL 3.8 caused the production of IL-2 (27). The transfection system presented here allows us to determine unequivocally whether there is a necessity for cellular presentation of the FL-Polymer in order to stimulate the transfectant to produce IL-2. Since the TCR β1 (Table 2) reconstituted 31-13 cells make IL-2 (41, 43), we first determined whether the αβFL cell line would also make IL-2 (Fig. 3 A) when challenged with a mAB against CD3 or with a combination of the calcium ionophore A23187 and PMA (44-46). We used a different αβFL cell line called 1C5 in this experiment (see Table 2 for a description of the transfectant cell lines), and found that 1C5 and Jurkat cells responded similarly to these stimuli. Subsequently, we incubated the cells shown in Fig. 3 B with FL-Polymers with and without PMA for 24 h, and quantitated the IL-2 production by CTLL assay. Clearly, the FL-Polymer in combination with PMA specifically activates the αβFL cell lines A5A6 and E3E4 (Table 2), although the FL-Polymer without PMA was not able to activate the αβFL T cells to make IL-2 (Fig. 3 B). Cell lines 3C11N and 31-13 did not respond to FL-Polymer, regardless of the polymer backbone (see Table 3). However 31-13 (Fig. 3 A) or 3C11N (data not shown) are capable of being activated to make IL-2 when incubated with calcium ionophore + PMA which bypasses the requirement for triggering through the surface TCR (42). These results explicitly show that only the αβFL transfectants will respond to FL-Polymers, whereas other T cells, although capable of making IL-2 (Figure 3 A) are incapable of functionally recognizing the FL antigen.
Inability to Detect MHC Class II Gene Expression in Transfected Cells by Northern Analysis. The direct binding studies described in the preceding section showed that FL Polymers which bind to the αFLβFL transfecants (Figure 2) could also functionally activate the T cells to produce IL-2. To determine whether the FL polymers were interacting with the TCR independent of MHC Class II molecules, we tested the transfecants for the expression of MHC Class II gene products by Northern analysis (Figure 4). We incubated a series of transfected cells with the polymer FL1000Dex2500 (Table 2) and PMA for 24 hours as we described earlier (Fig. 3 B). RNA was prepared from those cells and from control cell lines such as Jurkat, 31-13, and a EBV transformed autologous B cell line (RSEBV). The RNA was separated on a 1% agarose gel, and transferred to nitrocellulose, where it was probed with a combination of three MHC Class II cDNAs from DP, DQ and DR under normal stringency conditions at 42°C (50% formamide and 5x SSC hybridization buffer; 35). Only the RNA from the EBV transformed B cells had detectable MHC Class II mRNA expression (Fig. 4, lane 8), whereas none of the Jurkat derivatives including those which expressed the αFLβFL TCR had detectable MHC mRNA expression (Figure 4, lanes 1–7 and 9). Other Northern blots probed with each MHC Class II cDNA separately show an equivalent pattern of expression in the EBV transformed B cells as well as the Jurkat derivatives (Data not Shown). All of these Jurkat derivative cell lines express MHC Class I RNA in abundance (Data Not Shown), although Jurkat does not have any detectable CD8 gene expression (Figure 1 E, lane 8). These molecular analyses provide strong evidence that the interaction of the FL antigen with the TCR is independent of the involvement of MHC Class II gene products. We provide further evidence below using a system of FL substituted Sepharose beads.

FL Substituted Sepharose Beads Activate αFLβFL Transfected T Cells without PMA. It was apparent that in order to develop a system where the FL antigen directly interacted with T cells, in the absence of other stimuli or signals, it would be necessary to eliminate the need for PMA. It was previously shown that Sepharose 4B beads substituted with FITC could stimulate the T cell clone RFL 3.8 to secrete IL-2 in the absence of accessory cells or exogenous IL-2 (27). We reacted Sepharose 4B beads with FITC (FL Beads), incubated them with transfected cell lines for 24 h, and assayed supernatants for IL-2. The results are shown in Fig. 5 B. The FL Beads were fully capable of activating the αFLβFL cell line without
Figure 5. FL-derivatives: activation properties on αβ⁺ T cell transfectants. (A) Summary of FL-derivatives used in the analysis. The compounds shown in (A) were reacted with CNBr Activated Sepharose 4B with (Spacer) or without previous reaction with 1,6 diaminohexane. In (Cand D) all of the even numbers correspond to 1 mM of FL derivitization, and the odd numbers to 8 mM. In (C) all of the compounds differ from FITC in the "bottom" ring, while in (D) the "top" ring is substituted. (B) The indicated cell lines were mixed with FITC substituted Sepharose 4B beads containing the 1,6 diaminohexane spacer group. Other treatments such as PMA (P), Medium, or FL Polymer +P are for comparison purpose, and were done simultaneously with the other treatments. Refer to Fig. 3 A and Materials and Methods for details of the methods. (C) The indicated cell lines were treated with derivatized Sepharose 4B beads as shown in (A). The first series of bars represents results obtained with ethanolamine-substituted Sepharose 4B beads. (D) same as (C), except that the last series of bars represent results obtained with cells cultured with medium (M), or FL Polymer (FL).
PMA, and to a level that is comparable to the FL-Polymer and PMA treatment of the same cell line (Fig. 5 B). The degree of activation in most experiments is dependent on the concentration of FL on the bead, as exemplified in two separate cases where the Sepharose beads are reacted with 8 mM FL derivative, and they are almost twice as effective as those reacted with 1 mM FL (compare lanes 4 and 6 with 5 and 7 in Fig. 5 C). We have also found that FITC-derivatized APC (an autologous EBV-transformed B cell line) are able to specifically stimulate \( \alpha_\text{FL} \beta_\text{FL} \) T cell transfectants such as E3E4, but not control cell lines such as 31-13 or 3C11N (data not shown). Therefore, the \( \alpha_\text{FL} \beta_\text{FL} \) transfectants are able to recognize FL in the context of an APC, although there is no mandatory requirement for APC participation in the recognition complex of TCR + FL.

Fine Specificity of the \( \alpha_\text{FL} \beta_\text{FL} \) TCR for FL Derivatives. We took advantage of the ability of FL derivatized Sepharose beads to stimulate the \( \alpha_\text{FL} \beta_\text{FL} \) transfectants to explore further the specificity of the TCR for derivatives of FL (Figure 5 A). We coupled various derivatives of FL to Sepharose directly using available primary amines, or reacted the isothiocyanate forms of the FL derivatives to 1,6 diaminohexane substituted Sepharose (Figure 5 A). We then treated three different cell lines with the derivatized beads for 24 hours, and measured IL-2 in the supernatants using a CTL assay. The results are presented in Figures 5 C and D. We find a remarkable specificity of the TCR such that the mere alteration of the "bottom" ring substituent from 5' to the 6' position renders the compound almost completely unable to induce T cell activation. More radical substitutions to the triple ring obliterate any activity as shown in Figure 5 D. Only FL-Cad with its relatively long pentyl group was effective in activation, although the ethyl form (Figure 5 C, treatment 8, 9) was ineffective. In an effort to predict the importance of the N-C = S linkage to activity, we used derivatives which had an oxygen substituted for the sulphur (Figure 5 C, treatment 10-13). To our surprise both compounds were completely inert to activation of the \( \alpha_\text{FL} \beta_\text{FL} \) cells. These results show the exquisite specificity of the TCR for a particular antigen conformation and structure, and illustrate the complexity of studying interactions of more complex soluble antigens with TCRs.

Discussion
Recognition of antigen by T cells has been shown by a variety of methods to be restricted by the gene products of the MHC (see Introduction and reviewed in 44 and 14). It is generally accepted that a particular antigen is recognized by T cells only if processed fragments of the antigen bound to an MHC molecule are recognized by the TCR. Several studies have addressed the question as to whether a hapten can interact with TCR without MHC involvement. The hapten HSAB coupled to polyacrylamide beads was able directly to stimulate a murine T cell hybridoma, and the response could be effectively competed by soluble monomeric hapten (24). Other studies showed that the hapten TNP (47) and p-ABA (23) conjugated to a variety of proteins were capable of binding and activating specific T cell clones. The murine \( \gamma\delta \) gene segment in combination with different \( \beta \) gene segments was found to be capable of imparting specificity for p-ABA substituted proteins (48). Finally, in related studies using a chimeric immunoglobulin-TCR protein, it has been elegantly shown that the haptenic antigen coupled to Sepharose will activate the transfectant to make IL-2 (49).

Although the studies cited above indicate that hapten and hapten-protein conjugates can interact with TCR, the role of MHC molecules in these interactions has not been fully clarified. To address this concern, we have analyzed FL/TCR interactions in a system devoid of the relevant MHC molecules. Several lines of evidence suggest that FL can interact with the \( \alpha_\text{FL} \beta_\text{FL} \) TCR from a CD4+ FL-specific T cell clone in a functionally significant manner even in the absence of Class II MHC gene products. First, \( \alpha_\text{FL} \beta_\text{FL} \) transfectants that do not express detectable Class II MHC mRNA, bind FL-Polymers in a highly specific manner. Second, in the presence of PMA, FL-Polymers activate \( \alpha_\text{FL} \beta_\text{FL} \) transfectants, but not other T cells to produce IL-2. Cross-linking of TCR is important in this response, since monomeric FL is incapable of activation, although it can serve to a limited extent as a competitive inhibitor of FL-Polymer binding (27 and unpublished results). Third, FL derivatized Sepharose beads activate the \( \alpha_\text{FL} \beta_\text{FL} \) transfectants in a manner that shows exquisite antigen specificity (see below). This effect is mediated by the interaction of a matrix of FL groups with multiple TCRs on the T cell. Although physiologic antigen recognition clearly involves MHC presentation of processed antigen, our results conclusively demonstrate that hapten specific T cells can be activated by appropriate antigen-TCR interactions in the complete absence of the relevant class of MHC molecules.

Although the Jurkat transfectants described here express class I MHC gene products, a previous analysis with blocking antibodies indicate that class I molecules expressed by the T cell have no direct role in FL binding or in subsequent activation events. Studies with FL-specific T cell clones have shown that binding of FL polymers is completely unaffected by the presence of saturating concentrations of anti-class I antibodies (27). Even in the case of the FL-specific clones RFL 3.45 and RFL 1.65 which are CD8+ and class I restricted, blocking experiments with the anti-class I antibody W6/32 showed absolutely no inhibitory effect. Thus, class I is not required for FL binding to the T cell receptor on these cell lines. This is consistent with the exquisite specificity of FL binding (Figure 5). Only FL-specific clones and not other clones with the same class I MHC genotype bind FL (27). Furthermore, as shown here, only those transfectants with an appropriate combination of T cell receptor \( \alpha \) and \( \beta \) chains show FL binding even though all express the same complement of class I MHC molecules (Figure 2). The class I independence of the antigen binding of FL-specific T cells is further supported by the ability of solubilized RFL 3.8 T cell receptor molecules to bind to FL columns which do not significantly bind class I MHC molecules (27).

We have taken advantage of the direct interaction of antigen with TCR to study the fine specificity of the TCR.
for derivatives of FL. Our main conclusions are that the $\alpha_{FL}\beta_{FL}$ TCR is sensitive to any alteration of the "top" (triple ring) or "bottom" (single ring) of the FL structure (Figures 4A-D). The exchange of the isothiocyanate group from the 5' to the 6' position effectively disables the FL moiety from being an activator (Figure 5 C, 2-3). Additionally, the substitution of an amide (N-C-C) for a thiourea (N-C-N) linkage also eliminates recognition of the compound, with the caveat that the aminothioacetyl derivative of FL also differs slightly from the other methylene containing FL compounds in the spacer region (compare Figure 5 C 4-9 and 10-13). The relatively minor changes in the 5' bottom ring substituent (Figure 5 C, 10-11) completely abolishes the activating function. These data suggest that the single atomic substitution of an oxygen for a sulphur prevents the molecule from serving as an effective hapten for the $\alpha_{FL}\beta_{FL}$ TCR under study.

It was not clear from examining the FL derivatives such as 5(aminoacetamido)FL and FL-Cad whether the length of the methylene spacer group (–CH₂–) between the Sepharose bead and the FL moiety was a major factor in the activity of the FL derivative (Figure 5 C compare 4-9). We examined this issue further by comparing the activities of three FL derivatives that were bound to Sepharose by methylene containing spacer groups. FITC was reacted with diaminohexane substituted Sepharose resulting in a 6 carbon spacer group, whereas FL-Cad was directly reacted with the Sepharose producing a 5 carbon spacer. Both of these Sepharose derivatives were very active in stimulating the cell line E3E4 to make IL-2 (Figure 5 C, 4-5, 6-7). However, the 5((2-aminoethyl)thioureidyl)FL which contains only a 2 carbon spacer was not effective as an activating agent (Figure 5 C, 8-9). The original T cell clone RFL 3.8 was derived by stimulation with FITC derivatized cells on which FL groups were attached primarily to ε amino groups of lysine residues in membrane proteins. Therefore, it is not surprising that the TCR recognizes FL beads with 5 and 6 carbon spacers.

We can infer from our results that the $\alpha_{FL}\beta_{FL}$ TCR makes multiple contacts with several different atoms of the FITC molecule. Since FL is a rigid planar molecule there are no conformational isomers. This may explain how small haptens tethered to insoluble matrices are able to stimulate T cells, whereas peptides of sufficient size to contain activating epitopes possess too many conformational isomers that are not recognized by the highly-specific TCR. In fact, the crystal structure of the MHC Class I molecule (50) which shows a cleft that could bind peptides helps explain the ability of MHC-bound peptides to act as T cell activators. The peptide situated in the MHC cleft may be restricted to a limited number conformations, and as a result it could be expected to spend more time in conformations that are recognized by the TCR. Similarly, the same peptide that is recognized by a particular T cell in the context of one Class II MHC molecule, generally shows no activity when bound to APC with a different MHC haplotype. This has been elegantly shown for specific cytochrome C peptides and Class II MHC molecules in the mouse (12). Therefore, without MHC involvement, peptides generally are thought to be incapable of directly stimulating antigen-specific T cells.

One consequence of the requirement for a three way peptide + MHC + TCR interaction is that mutations in any of the components presumably affect the relationships of all the components. The FL system may circumvent this problem by eliminating the need for MHC interactions to obtain recognition of the haptenic antigen and the TCR. Future studies of this antigen: TCR interaction could be conducted on easily transfectable non T cell hosts (51), and a systematic mutagenesis of the TCR $\alpha$ and $\beta$ regions will be carried out utilizing the ability of the FL-Polymer to directly interact with the TCR (Buchwalder and Diamond, In Preparation). Creation of a phosphatidylinositol linked form of the $\alpha_{FL}\beta_{FL}$ TCR will allow us to prepare a soluble form using phospholipase C in order to study how the FL-Polymer might interact with a soluble TCR (51). These studies will lead to an understanding of how an antigen-specific TCR recognizes its ligand, and which amino acid side chains actually contact the FL molecule.

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