

# THE ANTIGEN-SPECIFIC, MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED RECEPTOR ON T CELLS

## VI. An Antibody to a Receptor Allotype

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A number of recent papers have described the properties of molecules on the surface of T cells that appear to be the receptors for antigen (Ag)<sup>1</sup> plus a product of the major histocompatibility complex (MHC) on these cells (1-6). In nearly every case these molecules were isolated using monoclonal antibodies prepared by immunizing mice or rats with cloned T cell lines or hybridomas. These monoclonal antibodies were found to be clone-specific, in that they reacted only with a glycoprotein on the surface of the T cells used for immunization and not with proteins on closely related T cells known to have different fine specificities for Ag/MHC. The clone specificity of these antibodies is, in fact, an important component of the evidence that they are indeed reacting with the Ag/MHC receptors on their target T cell clones and not some other surface molecule involved in T cell Ag recognition and activation.

While these antibodies have contributed tremendously to our understanding of T cell receptors, they have not been of much use in dissecting the events in the life history of the T cell *in vivo*, since they react with an undetectably low percentage of normal T cells. For these purposes an antibody directed against a nonidiotypic portion of the T cell receptor is needed. McIntyre and Allison (7) have described such a reagent in the shape of a rabbit antiserum against the receptor from a C57BL/6 T cell leukemia. The antiserum precipitates receptors from normal mouse thymocytes and peripheral T cells, and has been used to analyze constant and variable peptides in the glycoprotein (7). Recently, Bigler et al. have described the production of a monoclonal antibody to a human T cell leukemia that seems to cross-react with ~2% of human peripheral T cells (8). Since analysis with monoclonal antibodies is considerably easier than with whole antisera, we set out to make a similar reagent against mouse T cell receptors.

This paper describes the production and some of the properties of such an

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<sup>1</sup> *Abbreviations used in this paper:* Ag, antigen; FL, fluorescein; HgG, human gamma globulin; IL-2, interleukin 2; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; SN, supernatant; TGAL, poly L (Tyr, Glu)-poly-D,L-Ala-poly-L-Lys.

antibody. The monoclonal antibody, KJ16-133, was produced by immunizing a rat with the Ag/MHC receptor from a T cell hybridoma DO-11.10, specific for chicken ovalbumin and I-A<sup>d</sup> (OVA/I-A<sup>d</sup>, 9). The antibody inhibits Ag/MHC recognition by DO-11.10 and several other T cell hybridomas to which it binds, but stimulates others also bearing the determinant it recognizes and has no effect on Ag/MHC recognition by T cell hybrids to which it does not bind. KJ16-133 precipitates the 85-kd heterodimeric, Ag/MHC receptor from DO-11.10 and several other T cell hybridomas, including 3DT-52.5, a T cell hybridoma specific for D<sup>d</sup> (10). The monoclonal antibody precipitates similar molecules from peripheral T cells of most but not all strains of mice. Fluorescence studies showed that KJ16-133 detected the receptor on ~20% of peripheral T cells. Thus, this antibody detects an allotypic determinant present on some but not all T cell receptors.

### Materials and Methods

*Production and Characterization of T Cell Hybridomas Bearing Ag/MHC Receptors.* T cell hybridomas were produced as previously described by fusing Ag-primed, murine T cell blasts to the AKR thymoma BW5147 with polyethylene glycol (9–11). The Ags involved included chicken ovalbumin (cOVA, Sigma Chemical Co., St. Louis, MO), poly L (Tyr, Glu)-poly-D,L-Ala-poly-L-Lys (TGAL, Miles Laboratories, Inc., Elkhart, IN), human gamma globulin (HgG, Sigma Chemical Co.) and Keyhole limpet hemocyanin (KLH, Calbiochem-Behring Corp., American Hoeschst Corp., San Diego, CA). Hybrids were selected with hypoxanthine, aminopterin, and thymidine (HAT; Sigma Chemical Co.) and screened for the presence of receptors for Ag/MHC by their ability to secrete interleukin 2 (IL-2) in response to Ag and Ag-presenting cells of the appropriate H-2 type (11). These Ag-presenting cells were usually Ia<sup>+</sup> B cell lymphomas or hybridomas, but in some experiments fresh spleen cells were used (12). After the T cell hybridomas had been incubated for 24 h with Ag and Ag-presenting cells, supernatants (SNs) were assayed for their IL-2 content by titrating their ability to support the growth of the IL-2-dependent T cell line, HT-2, as previously described (11). Serial twofold dilutions of the SNs allowed us to measure how much IL-2 was in each one, one unit of IL-2 being defined as the minimum amount required to keep ~90% of 4,000 HT-2 cells alive in 0.1 ml of culture medium for 24 h. Table II lists the T cell hybridomas used in this paper and also their sources and reactivities.

In some cases DO-11.10/S4.4, a subclone of DO-11.10 selected for high receptor expression by immunofluorescent staining with the clone-specific anti-receptor antibody directed at DO-11.10, KJ1-26 (2), sorted and recloned, was used instead of DO-11.10. DO-11.10/S4.4 bears about twice as much receptor/cell as DO-11.10. The two lines were used interchangeably for the experiments described in this paper.

*Animals.* BALB/cBy and SJL/J were obtained from The Jackson Laboratory, Bar Harbor, ME or were bred in our colony from breeding triplets obtained from this source. SJA/20 mice were bred in our colony from breeding triplets obtained from Dr. L. A. Herzenberg, Stanford University, Palo Alto, CA.

Outbred Sprague Dawley rats were purchased from local sources.

*Preparation of the Ag/MHC Receptor from DO-11.10.* The Ag/MHC receptor from DO-11.10 was isolated from NP-40 lysates of the cells (surface labeled with <sup>125</sup>I [13, 14]) using a clone-specific antibody, KJ1-26.1. The properties of this antibody have been described elsewhere; it reacts with an idiotypic determinant on the Ag/MHC receptors of DO-11.10 (2). The antibody was used in two ways to isolate receptors. In the first method, NP-40 lysates of DO-11.10 were incubated with KJ1-26 and the antigen-antibody complexes were precipitated with *Staphylococcus aureus* (15). After thorough washing this preparation was used to immunize rats. In the second method, KJ1-26 was coupled to Sepharose beads, which were then incubated with NP-40 lysates of DO-11.10. After thorough

washing, the receptor was removed from columns of these beads using 0.05 M diethylamine, pH 11.5. The eluate was immediately neutralized with Tris and used as described below.

*Production of Monoclonal Antibody to T Cell Receptors.* Rats were immunized subcutaneously with the complex of DO-11.10 Ag/MHC receptor, KJ1-26 and *S. aureus* every 2 or 3 wk. The first immunization was given in complete Freund's adjuvant, and later immunizations were in incomplete Freund's adjuvant. Each immunization consisted of between  $10^8$  and  $10^9$  cell equivalents of DO-11.10 receptor. After about five immunizations the rats began to make antibodies against the receptor on DO-11.10 and similar material on peripheral BALB/c T cells, as judged by immunoprecipitation. After eight immunizations one rat was rested for a month, followed by a final immunization with receptor from  $\sim 10^9$  DO-11.10 isolated using KJ1-26 beads and diethylamine (see above). The rat was immunized subcutaneously, intraperitoneally, and intravenously. 3 d later spleen and draining lymph node cells from this animal were treated with an anti-T cell antibody and guinea pig complement (16), and fused to the nonsecreting plasmacytoma P3-X63 Ag8.653/3. Hybrids were selected with HAT, and when grown up, their supernatants were assayed for anti-DO-11.10 receptor antibodies by ELISA (see below).

*ELISA for Anti-receptor Antibodies.* The receptor from DO-11.10 was isolated from anti-receptor coupled beads as described above. An amount equivalent to  $3 \times 10^6$  DO-11.10 cells was added to half the wells of a nitrocellulose ELISA plate (STHA09610, Millipore Corp., Bedford, MA) and incubated in the well for 1 h to allow binding of the protein to the nitrocellulose filter. Monitoring with radiolabeled receptor showed that nearly all the protein bound irreversibly to the filter. Control wells were left empty. After 1 h 100  $\mu$ l of fetal bovine serum was added to all wells to saturate the nitrocellulose binding sites. 1 h later the plates were thoroughly washed with balanced salt solution and blotted dry. 80  $\mu$ l of SNs to be tested for anti-receptor activity were then added to a receptor-bound well and, as a control, to a well to which no receptor had been added. The plates were incubated for 1 h at room temperature and then thoroughly washed by suction. 100  $\mu$ l of a 1:250 dilution of alkaline-phosphatase-coupled rabbit antibody to rat immunoglobulins (Sigma Chemical Co.) was then added for a further hour. After, again, thorough washing, an alkaline phosphatase substrate, *p*-nitrophenyl phosphate, was added in the appropriate buffer (17), and the plates incubated at 37°C for 20 min. Supernatants were then transferred to flat-bottomed plastic microtiter plates, and the amount of substrate converted estimated using an ELISA reader (Bio-Tek Instruments, Burlington, VT). Controls included assay of receptor containing wells and empty wells with culture medium instead of hybridoma supernatant or with KJ1-26, the mouse clonotypic antibody to DO-11.10 receptor. In the latter case alkaline phosphatase-coupled rabbit antibodies to mouse immunoglobulins were used instead of antibodies to rat immunoglobulins (Sigma Chemical Co.).

*Preparation of T Cells.* T cells were prepared from the spleens and lymph nodes of normal animals by treating the cell suspensions with Gey's solution (18) followed by incubating them on, and eluting them from, nylon fiber columns (19).

*Surface Labeling and Lysis of Hybridoma or Normal T Cells.* Cells were labeled with  $^{125}\text{I}$  using iodogen and standard techniques (13, 14). After thorough washing they were lysed with 0.5% NP-40 in phosphate-buffered saline and insoluble material was removed by centrifugation.

*Immunoprecipitations.* Immunoprecipitations were done in several ways. Several of the mouse monoclonal antibodies used in this paper (KJ1-26, KJ12-98) have been purified and coupled to Sepharose beads. These were used to isolate material binding to these antibodies. The rat antibodies used in these studies, T24/40.7 (rat anti-mouse Thy-1.2, the kind gift of Dr. Ian Trowbridge, Salk Institute, La Jolla, CA) and KJ16-133, were incubated with rabbit or goat anti-rat immunoglobulin (Rockland, Inc., Gilbertsville, PA) coupled to Sepharose beads. These beads were then thoroughly washed before use in isolating material from lysates. Bound receptor was eluted from beads by boiling in sample buffer (20) or with diethylamine followed by neutralization (see above).

*Gel Electrophoresis of Immunoprecipitates.* Samples were run under nonreducing or

reducing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (20).  $\alpha$ - and  $\beta$ -chains of the reduced T cell receptors were separated as previously described by nonequilibrium pH gradient electrophoresis (NEPHGE) (21, 22).

**Immunofluorescence Staining.** Cells were washed and stained in phosphate-buffered saline containing 1% FBS and 0.2% sodium azide (PBS:FCS:AZ). The cells were incubated with an appropriate dilution of the primary antibody (KJ16-133.18) at 4°C or 37°C for 20 min, at a cell concentration of  $10^7$ /ml. Control cells were incubated in diluent without the primary antibody. The cells were then washed twice and incubated for 15 min at 4°C with an appropriate dilution of an affinity-purified, fluorescein-conjugated, mouse anti-rat kappa chain monoclonal antibody, MAR-18.5 (FL-MAR-18, the kind gift of Drs. Larry Arnold, University N. Carolina, Chapel Hill, NC and Lewis Lanier, Becton Dickinson, Mountain View, CA). The cells were then washed twice and resuspended at  $10^6$  cells/ml. Staining with KJ1-26 was done with directly fluoresceinated F(ab')<sub>2</sub> KJ1-26 antibody (FL-KJ1). In this case cells were incubated at  $10^7$ /ml as above, but no secondary antibody was used.

In experiments in which the competitive binding of different antibodies was to be examined cells were preincubated with saturating amounts of the competing antibody for 25 min in PBS:FCS:AZ. They were then washed and stained under the same conditions with biotin-conjugated anti-Thy-1 (T24/40.7) or biotin-conjugated KJ16-133 followed by avidin coupled with fluorescein or stained directly with FL-KJ1-26.

**Cytofluorimetric Analysis.** The relative fluorescence intensities of individual cells were measured using the Cytofluorograf System 50-H (Ortho Diagnostic Instruments, Westwood, MA) equipped with a 5-W argon laser. Forward narrow angle light scatter was used as a second parameter to facilitate exclusion of dead and aggregated cells. Fluorescence intensity data is presented as the log of the integrated fluorescence signal.

The percentage of antigen-expressing cells was determined using the Model 2150 Computer data-processing system. Fluorescence histograms containing equal numbers of cells that were either unstained, stained with either the fluorescein-conjugated secondary antibody alone, or stained with both the primary and secondary antibody reagents, were constructed and superimposed. Using the data-processing system the control values were subtracted from the experimental values on a channel by channel basis. The percentage of cells positively stained was determined by integration of the area to the right of the point of intersection of the two curves.

## Results

**Production of a Monoclonal Antibody to the Receptor on DO-11.10.** Rat monoclonal antibodies were screened for binding to the receptor on DO-11.10 by ELISA as described in Materials and Methods. Of ~1,500 hybridomas screened, about 9 gave high ELISA readings when added to wells containing DO-11.10 receptor, and low readings in wells containing no receptor. These nine hybridomas were grown up in 10-ml bottles, and their supernatants screened for their ability to precipitate receptor-like proteins from DO-11.10 and from normal T cells from BALB/c mice, the strain from which the DO-11.10 receptor was derived. Anti-Thy-1 precipitations were used as controls. One of these hybridomas, KJ16-133, precipitated material running at 85 kd on nonreduced gels from both DO-11.10 and BALB/c T cells (Fig. 1). On reducing gels the material from both DO-11.10 and normal T cells gave a diffuse band running at ~43 kd (Fig. 1). These results suggested that KJ16-133 precipitated receptors from both DO-11.10 and at least a subpopulation of T cells in normal BALB/c mice. The specificity of KJ16-133 was therefore clearly different from that of KJ1-26, the clone-specific antibody directed against the Ag/MHC receptor on DO-11.10, since KJ1-26 did not react

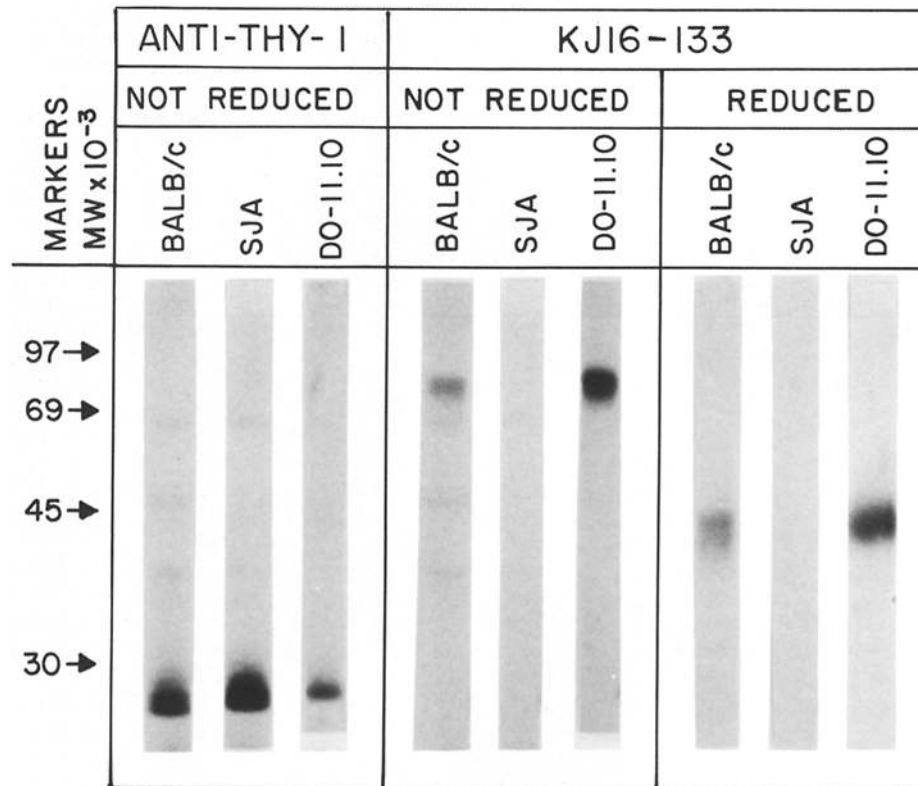


FIGURE 1. Immunoprecipitation with KJ16-133: normal T cells compared to DO-11.10. Normal T cells from BALB/c and SJA lymph node spleen were prepared. These hybridoma cells were surface labeled with <sup>125</sup>I. Lysates of the three cell preparations were incubated with Sepharose beads coupled with rabbit anti-rat IgG and preloaded either with rat anti-Thy-1 monoclonal antibody or with KJ16-133. The immunoprecipitates were subjected to SDS-PAGE under nonreducing conditions using a 10% acrylamide gel. The gel was stained, destained, and dried and an autoradiogram was prepared (left and middle panels). For the KJ16-133 immunoprecipitates, the region of the dried gel corresponding to molecules of 70–90,000 mol wt was cut out, rehydrated with Laemmli sample buffer containing 5% 2-mercaptoethanol, and subjected again to SDS-PAGE as before. The gel was stained, destained, and dried and an autoradiogram was prepared (right panel).

with a detectable percentage of BALB/c T cells (23, and unpublished data) nor did it precipitate receptor-like material from these cells (data not shown). KJ16-133 was cloned, and its clones assayed for anti-receptor activity by ELISA. All clones tested were positive and one, KJ16-133.18 was chosen for further use. This clone, the SNs of which were used for all the rest of the experiments described in this paper will be referred to as KJ16-133.

Interestingly, in the same experiment, KJ16-133 failed to precipitate receptor from SJA T cells, although these cells reacted normally with the anti-Thy-1 reagent. This result will be explored in greater detail below.

*Distribution of KJ16-133 Material on Different T Cell Hybridomas.* The fact that KJ16-133 precipitated a significant receptor band from normal BALB/c T cells suggested that the antibody was recognizing a determinant present on a signifi-

cant percentage of T cell Ag/MHC receptors. KJ16-133 was therefore screened for its ability to precipitate receptor material from a number of T cell hybridomas. In each case,  $^{125}\text{I}$  surface-labeled lysates from the T cells were incubated with Sepharose beads covalently coupled to rabbit anti-rat immunoglobulin and then bound with KJ16-133. Controls (not shown here) included precipitations from the same cells with rat anti-mouse Thy-1.2 antibody, or with rabbit anti-rat immunoglobulin-coupled beads not coated with a second antibody.

After thorough washing, bound material was eluted using SDS and run on nonreducing SDS-PAGE. Some sample gels are shown in Fig. 2. KJ16-133 bound receptors from SKK-45.10 and DIG10G11 but not from a number of other T cell hybridomas tested, including one of BALB/c origin, 3DT-18.11.

In some cases hybrids were screened for reactivity with KJ16-133 by ELISA. Some sample data are shown in Table I. The antibody reacted well with five of the hybrids listed, including SKK-9.11 and 3DT-52.5, but not at all with a number of other cell lines.

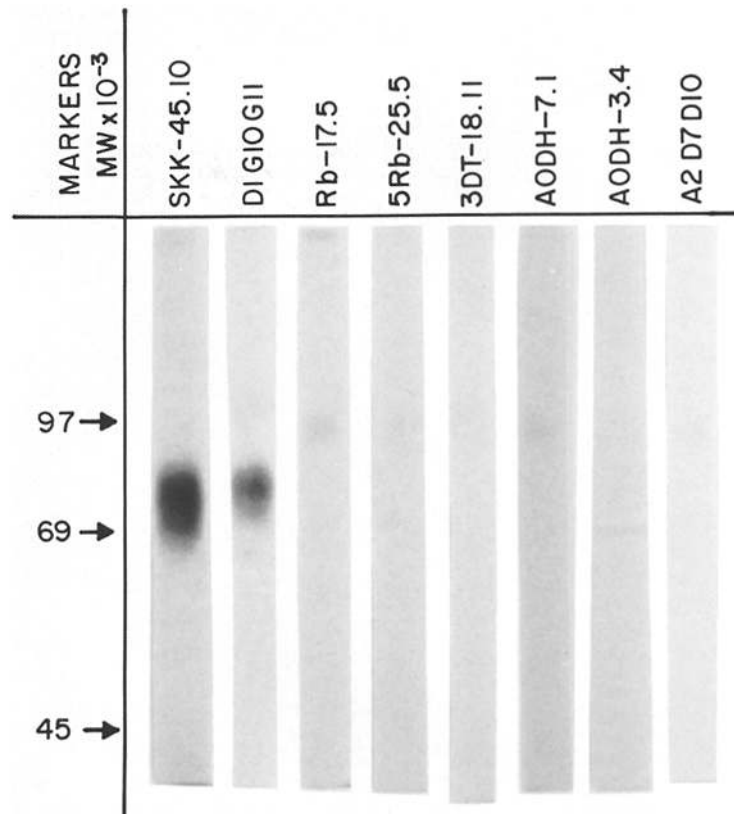


FIGURE 2. Immunoprecipitation with KJ16-133: comparison among a bank of T cell hybridomas. Eight T cell hybridomas were surface labeled with  $^{125}\text{I}$ . A lysate of each hybridoma preparation was incubated with Sepharose beads coupled with rabbit anti-rat IgG and preloaded with KJ16-133. The immunoprecipitates were subjected to SDS-PAGE under nonreducing conditions using a 7.5% acrylamide slab gel. The gel was stained, destained, and dried and an autoradiogram was prepared.

TABLE I  
Reactivity of KJ16-133 with T Cell Hybridomas and Tumors

Cell line	ELISA OD ( $\times 1000$ ) with*:	
	Anti-Thy-1.2	KJ16-133
D0-11.10/S4.4	302 $\pm$ 14	146 $\pm$ 7
3DT-52.5	557 $\pm$ 34	174 $\pm$ 10
D1G10G11	468 $\pm$ 9	101 $\pm$ 8
SKK-9.11	543 $\pm$ 13	79 $\pm$ 6
SKK-45.10	637 $\pm$ 22	58 $\pm$ 3
3D0-20.10	530 $\pm$ 13	5 $\pm$ 10
SKK-2.3	478 $\pm$ 19	-3 $\pm$ 4
SKK-9.19	599 $\pm$ 10	9 $\pm$ 5
A0DH-3.4	467 $\pm$ 26	-12 $\pm$ 8
C6VL-1	388 $\pm$ 14	13 $\pm$ 10

\* ELISA readings were calculated by averaging triplicate wells, subtracting background readings from wells incubated with cells and alkaline phosphatase-coupled rabbit anti-rat immunoglobulin but no primary antibody, and combining the errors appropriately to obtain standard errors for each corrected reading.

TABLE II  
Origins and Reactivities of T Cell Hybridomas and Tumors

Cell line	Origin*	Specificities	Reaction with KJ16-133	Reference
D0-11.10	BALB/c	cOVA/I-A <sup>d</sup> ; I-A <sup>b</sup> ; cOVA/I-A <sup>b</sup>	+	9
3D0-54.8	BALB/c	cOVA/I-A <sup>d</sup> ; I-A <sup>s,f,k</sup>	-	24
3D0-18.3	BALB/c	cOVA/I-A <sup>d</sup>	-	24
3D0-20.10	BALB/c	cOVA/I-A <sup>d</sup>	-	24
3DT-18.11	BALB/c	TGAL/I-A <sup>d</sup>	-	9
3DT-52.5	BALB/c	D <sup>d</sup>	+	10
MDK16	BALB/c	I-A <sup>k</sup>	+	—
S18.4	BALB.k	I-E <sup>d</sup>	+	—
A2D7D10	C57BL/6	I-E <sup>d</sup> ; I-A <sup>b</sup>	-	—
D1G10G11	C57BL/6	I-A <sup>d</sup> ; M1s <sup>a,d</sup>	+	—
SKK-2.3	(SJA $\times$ C58)F <sub>1</sub>	KLH/I-A <sup>k</sup>	-	—
SKK-9.11	(SJA $\times$ C58)F <sub>1</sub>	KLH/I-A <sup>k</sup>	+	—
SKK-45.10	(SJA $\times$ C58)F <sub>1</sub>	KLH/I-A <sup>k</sup> ; I-A <sup>b</sup>	+	—
A0DH-3.4	B10.A	cOVA/I-A <sup>k</sup> ; I-A <sup>b</sup>	-	12
A0DH-7.1	DBA/2	HgG/I-E <sup>d</sup>	-	25
Rb-17.5	Rb(8.12)5Bnr	KLH/I-A <sup>b</sup>	-	26
5Rb-25.5	Rb(8.12)5Bnr	KLH/I-A <sup>b</sup>	-	26
WeHi7	BALB/c	Unknown	-	—
EL-4	C57BL/6	Unknown	-	—
C6VL-1	C57BL/Ka	Unknown	-	1
BW5147	AKR	Unknown	-	—

\* Origin of normal T cell parent donating the receptor of the hybridoma, or of the tumor.

The specificities and origins of all the T cell hybridomas we have tested so far for reaction with KJ16-133 in either of these assays are shown in Table II. The normal T cell parents of all the T cell hybridomas and tumors shown were derived from strains of mice known to express the KJ16-133-reactive determi-

nant. Clearly there is no obvious relationship between the specificities or origins of those T cell hybrids with which the antibody reacts. The antibody reacts with some but not all closely related hybrids; for example, it binds to DO-11.10, but not to 3DO-54.8, a T cell hybridoma of BALB/c origin that reacts with the same cOVA tryptic peptide as DO-11.10 plus I-A<sup>d</sup>. The unpredictable nature of the reaction of the antibody is also illustrated by 3DT-52.5, which is the only Class I-specific T cell hybrid we have isolated, and yet reacts with KJ16-133, derived from immunization with the receptor from a Class II-restricted hybrid. Reactivity is not limited to T cells specific for antigen plus MHC; some alloreactive hybrids also bind the antibody, for example S18.4. Of potential future interest, D1G10G11, a hybrid with reactivity for either I-A<sup>d</sup> or Mls<sup>a,d</sup>, binds the antibody.

*The Clone-specific Antibody KJ1-26, and KJ16-133 Precipitated the Same Material.* We wished to confirm the specificity of KJ16-133 for T cell receptors by demonstrating that it, and the clone-specific antibody, KJ1-26, precipitated the same material from DO-11.10. This was demonstrated by the experiments shown in Fig. 3.

DO-11.10 <sup>125</sup>I-labeled lysate was immunoprecipitated with KJ1-26 or with KJ16-133 or anti-Thy-1.2 absorbed to rabbit anti-rat immunoglobulin coupled to Sepharose 4B beads. Supernatants of these immunoprecipitates were then divided in half, and one-half incubated with KJ1-26 and the other half with KJ16-133. Bound material from all these reactions was then run on SDS-PAGE nonreducing gels. As shown in Fig. 3, KJ1-26 and KJ16-133 both precipitated material running at 85 kd from DO-11.10. Neither KJ1-26 nor KJ16-133 could precipitate more material from the SNs of these incubations. Both precipitated 85-kd material from the supernatants of anti-Thy-1 incubations. Thus, the targets of KJ1-26 and KJ16-133 on DO-11.10 are identical.

*Effects of KJ16-133 on the Response of Target T Cell Hybridomas.* KJ1-26, the clone-specific antibody against the receptor for OVA/I-A<sup>d</sup> on DO-11.10, inhibits production of IL-2 by DO-11.10 in response to OVA/I-A<sup>d</sup> (2). In aggregated form, KJ1-26 stimulates IL-2 production by DO-11.10 in the absence of other stimuli (22). Similar effects have been seen by us and others working with other T cells and their clone-specific antibodies (4-6). We were not sure that KJ16-133 would have comparable effects. The antibody is not directed at an idiotypic portion of the receptor, i.e., it may not interfere with Ag/MHC binding and therefore might not inhibit T cell responses to this combination. Nevertheless, a number of hybridomas known to react with KJ16-133, and a control hybridoma known not to bind the antibody (3DO-54.8), were incubated with or without their specific targets in the presence or absence of KJ16-133 antibody, and its effects on their responses monitored. Some typical results are shown in Table III. KJ16-133 blocked the responses of DO-11.10, SKK-9.11, and D1G10G11 rather well. Interestingly, in cases in which the hybridoma tested was known to have more than one reactivity, all were inhibited by the antibody. Thus, KJ16-133 inhibited the response of DO-11.10 to both cOVA/I-A<sup>d</sup> and cOVA/I-A<sup>b</sup> and the response of D1G10G11 to both I-A<sup>d</sup> and Mls<sup>a</sup> although this last inhibition was variable and sometimes marginal. The implications of this finding will be examined in a later paper. The antibody inhibited the response of SKK-45.10 to KLH/I-A<sup>k</sup> less strongly. SKK-45.10 responds very well to KLH/I-A<sup>k</sup>, reacting at



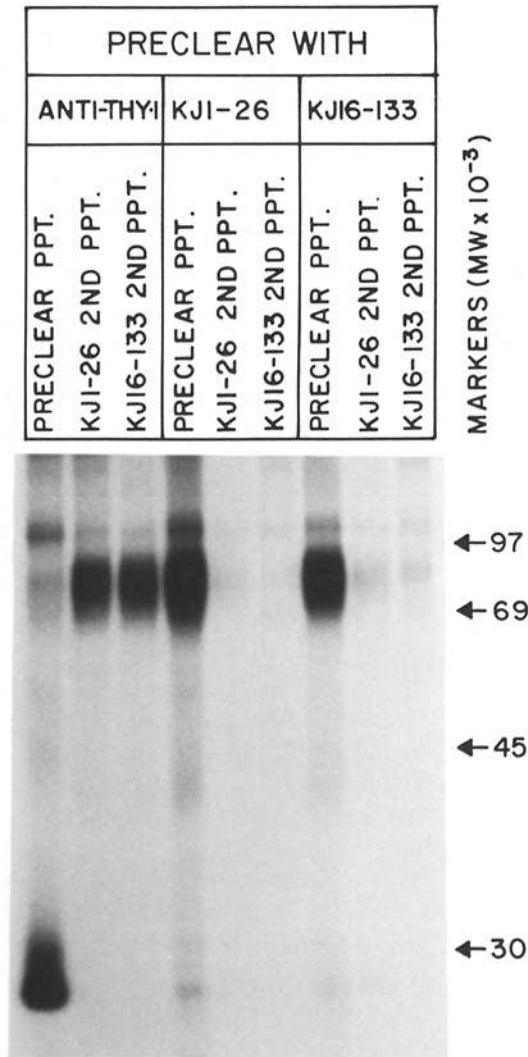


FIGURE 3. KJ1-26 and KJ16-133 detect the same molecule on DO-11.10. DO-11.10 hybridoma cells were surface labeled with <sup>125</sup>I. Three aliquots of the cell lysate were precleared by immunoprecipitation using one of three Sepharose preparations: (a) beads coupled with rabbit anti-rat IgG and then preloaded with rat-anti-Thy-1 monoclonal antibody; (b) the same type of bead preloaded with KJ16-133; and (c) beads directly coupled with KJ1-26. In each case the immunoprecipitating antibody was calculated to be in excess in relation to the cell lysate. The supernatants from each of these first immunoprecipitations was split into two aliquots and a second immunoprecipitation performed with KJ16-133 and KJ1-26 as before. Both the first and second immunoprecipitates were subjected to SDS-PAGE under nonreducing conditions using a 10% acrylamide slab gel. The gel was stained, destained, and dried and an autoradiogram of the dried gel prepared.

low concentrations of KLH and with few Ag-presenting cells, suggesting that its receptors may have a very high affinity for KLH/I-A<sup>k</sup>. We suspect that the less striking effects of KJ16-133 on this hybridoma may be due to inability of the

TABLE III  
*Effects of KJ16-133 on the Responses to Ag/MHC of Different T Cell Hybridomas*

T cell hybridoma	Stimulus	U/ml IL-2 produced in the presence of:	
		No antibody	KJ16-133*
D0-11.10	cOVA/I-A <sup>d</sup>	320	<10
D0-11.10	cOVA/I-A <sup>b</sup>	160	<10
D0-11.10	None	<10	<10
SKK-9.11	KLH/I-A <sup>k</sup>	320	<10
SKK-9.11	None	<10	<10
D1G10G11	I-A <sup>d</sup>	80	<10
D1G10G11	M1s <sup>a</sup>	160	10
D1G10G11	None	<10	<10
SKK-45.10	KLH/I-A <sup>k</sup>	640	80
SKK-45.10	None	<10	<10
3DT-52.5	D <sup>d</sup>	40	320
3DT-52.5	None	<10	40
S18.4	I-A <sup>d</sup>	>700	240
S18.4	None	<10	80
3D0-54.8	cOVA/I-A <sup>d</sup>	640	640

\* 40% culture supernatant of the antibody-secreting hybridoma.

antibody to compete well with the reaction of these receptors with their Ag/MHC targets.

Perhaps surprisingly, this same soluble antibody preparation of KJ16-133 stimulated IL-2 production by two T cell hybridomas with which it could react, 3DT-52.5 and S18.4. This effect was not observed with any other of the hybrids we tested. It is difficult at this point to account for this result, except to suggest that different T cell hybrids may have different requirements for crosslinking their receptors before triggering the cell. As far as 3DT-52.5 is concerned, this hybridoma has some unusual properties that may have contributed to the effects of KJ16-133. 3DT-52.5 is specific for D<sup>d</sup>, a molecule it bears at low (substimulatory) levels on its own surface. Interaction with its own D<sup>d</sup> may, however, contribute to the stimulation seen when KJ16-133 is added to the cultures. KJ16-133 had little or no effect on the response to Ag/MHC of 3D0-54.8, a T cell hybridoma known not to react with the antibody.

Taken together, however, we feel that the results shown so far in this paper prove that KJ16-133 reacts with the receptors for Ag/MHC on the surfaces of some T cells and not with some other molecule that might be present on some but not all hybrids. The nature of the determinant recognized will be discussed below.

*KJ16-133-reactive Material on Normal T Cells.* Antiserum from the donor rat for the KJ16 fusion precipitated receptor-like material from normal BALB/c T

cells at the time cells were taken from the animal and, as shown in Fig. 1, the monoclonal antibody KJ16-133 reacted with at least some T cells in BALB/c mice. A more extensive survey of different mouse strains was therefore done by immunoprecipitation. The results of this survey, to be described in a later publication, were that KJ16-133 reacted with at least some T cells in most common laboratory strains of mice.

Further documentation of the finding that SJA-related T cells do not react with KJ16-133 is shown in Fig. 4. In this experiment KJ16-133-reactive material was precipitated from BALB/c or SJL T cells as described above and then run in the first dimension on nonreducing SDS-PAGE. The relevant strips were then cut out, turned sideways, and run reduced on SDS-PAGE in the second dimension. By such procedures BALB/c T cells yielded a band running at 80–85 kd nonreduced, and 40–45 kd reduced, as predicted for Ag/MHC receptors (1, 2). This band was not apparent on gels from SJL T cells.

KJ16-133 therefore reacts with the receptors on at least some T cells in most strains of mice, but the determinant recognized by the antibody is lacking in SJL and SJA animals. Several points concerning this result can be made here. The fact that BALB/c T cells bear this determinant but SJA T cells do not proves that the target of KJ16-133 does not map to the *Igh* locus, since both BALB/c and SJA are *Igh*<sup>a</sup>. Furthermore, the fact that SJA and SJL T cells are negative, but that B10.S(7R) T cells were found to bear the antigen (data not shown), suggests it does not map to the MHC. Finally, the fact that SJA and SJL T cells do not bear the antigen allows them to be used as useful negative controls in the cytofluorograph experiments described below.

*Distribution of KJ16-133-reactive Material on T Cells.* We wished to find out what percentage of BALB/c T cells reacted with KJ16-133. Preliminary experiments were done using conventional binding conditions for the antibody, i.e., incubation of T cells with saturating amounts of KJ16-133 followed by staining

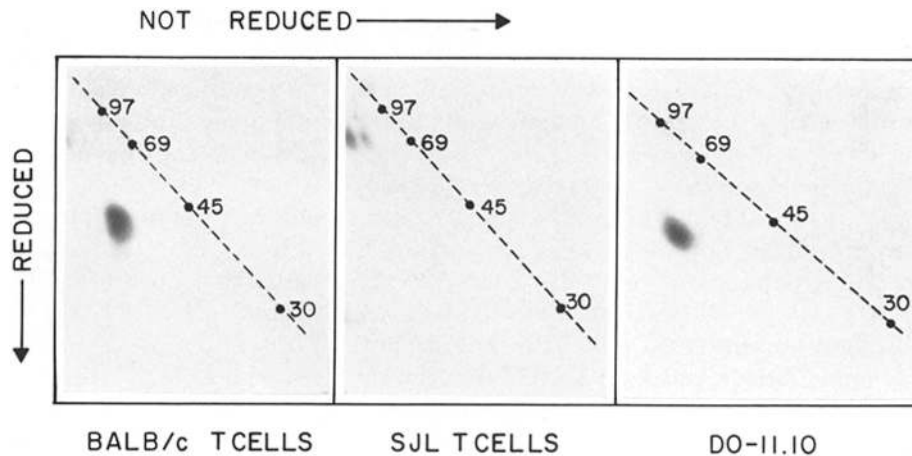


FIGURE 4. SJL T cells do not bind KJ16-133. SJL and BALB/c T cells and DO-11.10 were labeled with  $^{125}\text{I}$ , lysed, and reacted with KJ16-133 absorbed to goat anti-rat immunoglobulin-coupled beads. Bound material was run on 10% nonreducing SDS-PAGE. Strips containing these materials were then cut out and run in the second dimension on 10% reducing SDS-PAGE.

with fluorescein-coupled mouse anti-rat kappa chain antibody (FL-MAR18). These conditions gave a population of very faintly stained T cells. Several other protocols were therefore tried, including reaction of the T cells with KJ16-133 at 37°C in the presence of azide. This procedure stained T cells much more strongly. Comparison of 4°C and 37°C staining conditions for DO-11.10 gave the same result, i.e., KJ16-133 reacted well with the cells at 37°C and poorly at 4°C. Some examples of our results are shown in Fig. 5. BALB/c T cells (96% Thy-1<sup>+</sup>, data not shown) had some autofluorescence (data not shown). This profile was unaffected by incubation with FL-MAR18 save that a few cells stained weakly with FL-MAR18 at both 4°C (not shown) and 37°C (solid line, Fig. 5a). Reaction with KJ16-133 stained some BALB/c T cells; this was much more marked if the KJ16-133 incubation was carried out at 37°C rather than 4°C (compare the dotted and dashed lines, Fig. 5a). There was no staining above background of SJL T cells (97% Thy-1<sup>+</sup>, data not shown) under any conditions (Fig. 5b shows data with 37°C incubated cells). Integration of the areas under the curves shown in Fig. 5a, and subtraction of controls showed that KJ16-133 reacted with 19.5% of BALB/c T cells at 37°C and a similar percentage, though less strongly, at 4°C. Preliminary results suggested that this percentage of staining T cells is the same in other strains of mice that react with the antibody.

As mentioned above, the antibody showed the same temperature sensitivity in its reaction with the T cell hybridoma DO-11.10. 100% of these cells stained weakly with KJ16-133 at 4°C and strongly at 37°C (Fig. 5c). By contrast staining

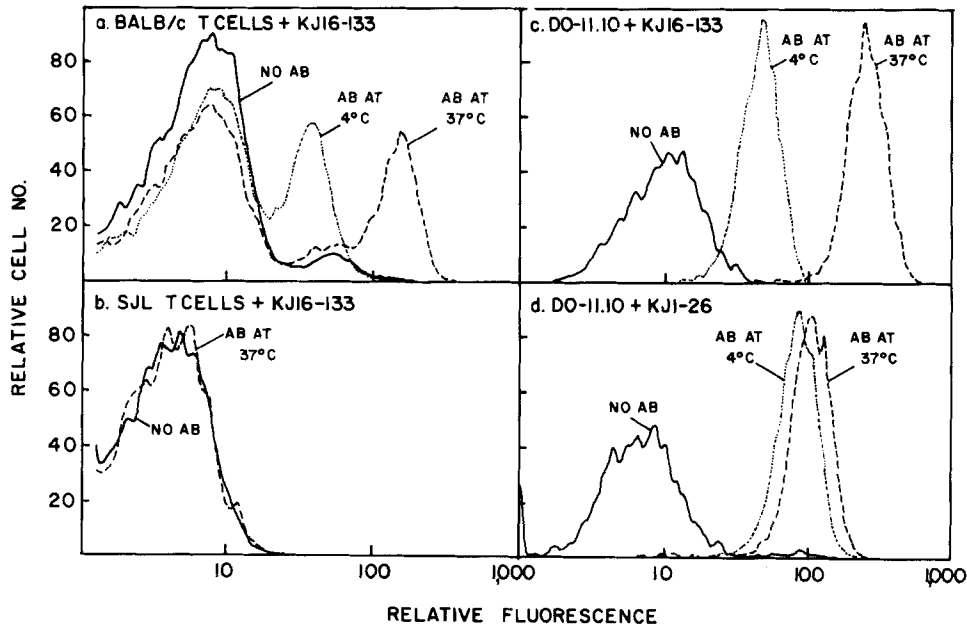


FIGURE 5. KJ16-133 stains only a portion of peripheral T cells in BALB/c mice. BALB/c or SJL T cells or DO-11.10 were stained with fluorescein-coupled monoclonal mouse anti-rat kappa chain (FL-MAR 18). In some cases these cells had been preincubated at 4°C or 37°C in azide with saturating amounts of KJ16-133. Cells were then thoroughly washed and analyzed for fluorescence staining using a cytofluorograph.

with the clone-specific antibody, KJ1-26, was relatively unaffected by the incubation temperature (Fig. 5d).

These results showed that the determinant recognized by KJ16-133 is present on ~20% of peripheral T cells in reactive mouse strains, and that it is apparently more readily exposed at physiological temperatures than in the cold.

*KJ16-133 Does Not Bind to the Same Determinants as a Clone-specific Antibody.* We wished to find out what portion of the T cell receptor was recognized by KJ16-133. The fact that the antibody does not react with all T cells proves that it does not recognize a determinant common to all receptors. We felt that three targets could therefore be suggested. KJ16-133 might bind a determinant on a family of V-regions, it might bind to one of several J regions, or it might bind to one of several constant regions that might be expressed by one of the chains of the T cell receptor. For example, Yanagi et al. and Hedrick et al. (27, 28) have recently described a cDNA clone that probably codes for one of the chains of the T cell receptor. It has been shown that these probes detect a family of genes including an unknown number of V regions, more than one expressed J region, and two closely related but not identical constant regions (28, 29). Thus, any of the possibilities suggested above as targets for KJ16-133 would be supported by what is known from molecular biological studies.

First we tried to find out whether KJ16-133 would bind to one of the separated chains of the T cell receptor. After the chain had been reduced and separated by either NEPHGE in 9 M urea or incubation at room temp with 1% SDS, neither KJ16-133 nor KJ1-26, the clone-specific antibody reacted with the receptor from DO-11.10 (data not shown). Since these methods involve harsh denaturation procedures, we decided to try a different approach in which the chains were reacted with the antibodies whilst they were still more or less native. The receptor was therefore isolated from <sup>125</sup>I-surface labeled DO-11.10 by binding to KJ1-26 beads, elution with diethylamine, and rapid neutralization. Half of this sample was then reduced with 0.03 M dithiothreitol and alkylated with excess iodoacetamide. We then tested the ability of KJ16-133 and the clone-specific antibody, KJ1-26, to reprecipitate the receptor. The precipitated material was analyzed for its content of  $\alpha$  and  $\beta$  chains by NEPHGE.

The results of this experiment are shown in Table IV. KJ1-26 and KJ16-133 were able to reprecipitate an equal quantity of unreduced receptor. Not surprisingly, the precipitated material contained both  $\alpha$ - and  $\beta$ -chains (in DO-11.10 the  $\alpha$ -chain is usually somewhat more heavily labeled with <sup>125</sup>I than the  $\beta$ -chain). Reduction and alkylation did not affect the ability of KJ16-133 to precipitate both the  $\alpha$ - and  $\beta$ -chains of the receptor; however, precipitation by KJ1-26 was dramatically lowered by this procedure.

From this experiment we concluded that KJ1-26 and KJ16-133 were recognizing different determinants on the receptor. We could not tell whether or not KJ16-133 would recognize isolated, native  $\alpha$ - or  $\beta$ -chains, since both were precipitated by the antibody, probably because the alkylation and reduction procedure under native conditions did not separate them.

A second type of experiment was used to show that KJ1-26 and KJ16-133 bind different parts of the receptor molecule. DO-11.10 cells were preincubated with saturating concentrations of anti-Thy-1 or KJ1-26 at 4°C, or KJ16-133 at 37°C

TABLE IV  
Sensitivity of Determinants on the Receptor to Reduction and Alkylation

Treatment of the receptor of D0-11.10 purified with KJ1-26 antibody		Total	Immunoprecipitated cpm* recovered in:	
Reduction and alkylation	Reprecipitated with		$\alpha$ -Chain	$\beta$ -Chain
–	KJ1-26	1,500	510	357
+	KJ1-26	300	70	70
–	KJ16-133	1,650	587	325
+	KJ16-133	1,700	490	490

Receptor was immunoprecipitated from lysate of  $^{125}\text{I}$ -labeled D0-11.10 cells using KJ1-26 antibody coupled to Sepharose beads. The bead-bound receptor was eluted with 0.05 M diethylamine (DEA), neutralized, aliquoted, and lyophilized. Four aliquots containing 9,000 cpm each were resuspended in 0.5 ml of 0.05 M DEA containing 0.5% NP-40. Two samples were made 30 mM in DTT. After 15 min at room temperature all samples were made 50 mM in IAA. Reduced and alkylated samples were neutralized with 0.05 M DEA. Nonreduced samples were neutralized with  $\text{CO}_2$ . Each sample received 1.5 ml of standard lysis buffer containing PBS, 1% NP-40, 50 mM IAA, and  $10^{-4}$  M PMSF and was subjected to immunoprecipitation with either KJ1-26- or KJ16-133-coupled Sepharose beads. The bound receptor was then again eluted with 0.05 M DEA and subjected to NEPHGE analysis to separate  $\alpha$ - and  $\beta$ -chains. Shown are the cpm eluted from the second immunoprecipitation and the cpm recovered in the  $\alpha$ - and  $\beta$ -containing fractions of the NEPHGE gels.

in PBS:FCS:AZ. The cells were then washed and incubated with FL-KJ1-26 or biotin-conjugated anti-Thy-1 (4°C) or biotin-conjugated KJ16-133 followed by fluorescein-conjugated avidin, again in PBS:FCS:AZ. The Cytofluorograf was then used to assess how well the antibodies competed with each other for binding to DO-11.10. The averaged results of three experiments are shown in Fig. 6. Preincubation with each antibody very successfully blocked subsequent binding of the same antibody. Preincubation with anti-Thy-1 had little effect on the subsequent binding of KJ1-26 or KJ16-133. Likewise, KJ1-26 and KJ16-133 did not block binding of anti-Thy-1. Significantly, KJ16-133 did not block binding of KJ1-26, and KJ1-26 preincubation only inhibited by 50% the subsequent binding of KJ16-133. The 50% inhibition of KJ16-133 binding by KJ1-26 preincubation, in the absence of significant inhibition when the experiment was done the other way around, could have occurred for several reasons. The most likely explanation is that KJ1-26 was able to inhibit sterically the binding of the bulky complex of biotinylated KJ16-133 and fluorescein-conjugated avidin, whereas KJ16-133 was not able to hinder the binding of the smaller FL-KJ1-26. However, since no inhibition was seen in one combination, and only partial inhibition in the other, we concluded that KJ1-26 and KJ16-133 were probably binding to different regions of the receptor molecule.

Since KJ1-26 is known to bind to the idiotypic, antigen plus MHC binding site on the receptor (2, 23), we concluded from these experiments that KJ16-133 was probably binding to a different region, for example the constant region.

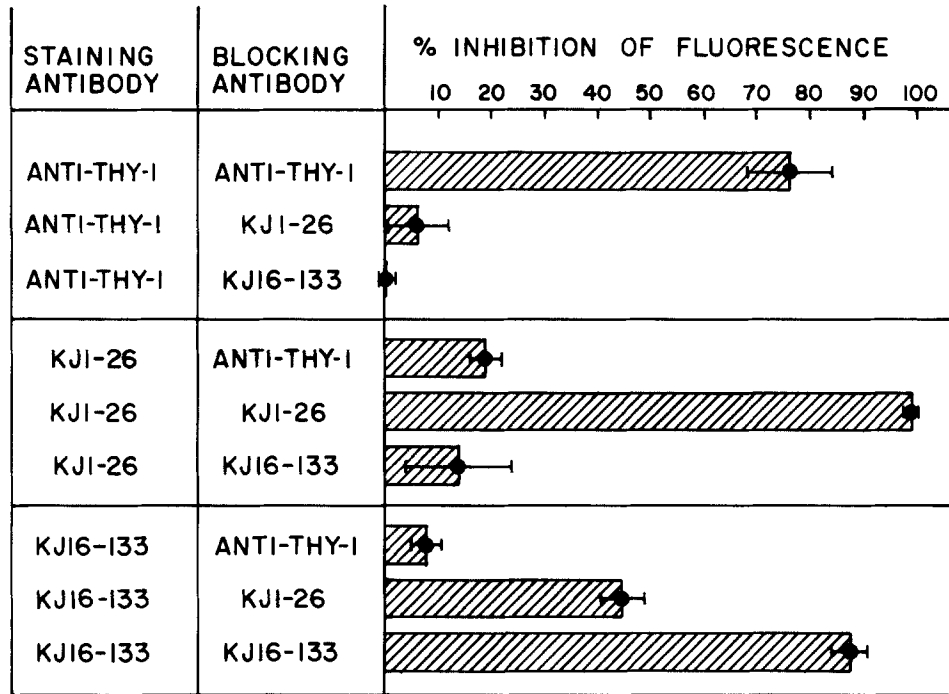


FIGURE 6. KJ16-133 and KJ1-26 bind different sites on the receptor. DO-11.10 cells were preincubated in PBS:FCS:AZ with saturating concentrations of anti-Thy-1 or KJ1-26 or KJ16-133 at 37°C. The cells were then washed and stained with FL-KJ1-26 or biotin-conjugated KJ16-133 or biotin-conjugated anti-Thy-1 at the appropriate temperatures. Cells stained with biotin-conjugated antibodies were reacted after washing with avidin coupled to fluorescein. Relative mean fluorescence was determined for the cells stained under each set of conditions using a Cytofluorograf System 50H and compared with controls incubated with no stain or with fluorescein-conjugated avidin alone. These two controls were identical. In the absence of blocking antibodies, 100% of the cells stained with each reagent. For each set of conditions the percent inhibition of immunofluorescent staining was calculated by the following formula: (mean fluorescence with blocking antibody minus background control) divided by (mean fluorescence without blocking antibody minus background control). The figure depicts the % inhibition of fluorescence  $\pm$  standard error of the mean of three identical experiments.

### Discussion

This paper describes the production and properties of a monoclonal antibody that recognizes the Ag/MHC receptor of some but not all murine T cells. It was produced in a rat immunized with isolated receptor from a cloned T cell hybridoma, DO-11.10, bound to its antiidiotypic antibody, KJ1-26, and *S. aureus*. Cross-reactive antisera were raised relatively easily in this rat; only about five immunizations were required. Interestingly, of the 1,500 hybridomas we assayed from this animal, none were secreting clonotypic antibodies, the type most usually raised when whole cell immunizations are used. This may be due to masking of clonotypic determinants on DO-11.10's receptor by KJ1-26.

The monoclonal antibody we obtained, KJ16-133, had several unexpected properties. First, it reacted with only a subpopulation of T cells from, for example, BALB/c mice. In repeated experiments the percentage of cells recognized was ~15–20%. This was apparent not only from staining data, shown in

this paper, but also from the results of immunoprecipitation experiments. Order-of-magnitude calculations about  $^{125}\text{I}$ -labeling of Ag/MHC receptors on T cells, assuming between 20,000 and 40,000 receptors/cell, had led us to expect that KJ16-133 would precipitate between 2 and 10 times more radioactivity in the receptor band from BALB/c T cells than it did were it to react with receptors on all T cells. The restricted reactivity of KJ16-133 was also, of course, manifest by its reactions with different T cell hybridomas and tumors. The antibody only reacted with ~25% of all T cell hybridomas we have tested. Even T cell hybridomas with closely related origins and specificities were not similarly reactive. DO-11.10 and 3DO-54.8, for example, were both derived from BALB/c T cells primed with cOVA, they recognized the same cOVA tryptic peptide plus I-A<sup>d</sup> (24), and yet the receptor of one of these hybrids reacted with KJ16-133, and the receptor of the other did not. In fact, even a cursory glance at the list of hybrids with which KJ16-133 does or does not react reveals that there is no obvious pattern to the determinant recognized by the antibody. It is present on both I-A and I-E restricted T cells. It is found on T cells that respond to Ag plus self-I, and on T cells specific for allogeneic I-A or I-E. It is present on the only Class I-reactive T cell hybridoma we have, 3DT-52.5. Thus, the determinant recognized here does not define a particular Ag or I specificity, indeed, it does not even distinguish between Class I- and Class II-reactive receptors. (In support of this conclusion, preliminary experiments have suggested that the determinant is present on both L3T4- and Lyt 2-bearing normal T cells.) In addition, experiments to be published shortly suggest that the determinant is present on subpopulations of cortisone-sensitive thymocytes, indicating that it is not a marker solely for late, fully mature T cells.

We have done a number of experiments to examine the relationship between the determinant recognized by KJ16-133, and that bound by the antiidiotypic antibody, KJ1-26. The clone-specificity and ability of the latter to define both antigen and MHC-reactivity (6, 23) both suggest that this antibody detects determinants in or close to the V, D, or J regions of the receptors (27-29). We found that KJ16-133 probably binds to some part of the molecule that does not involve these regions because KJ1-26 and KJ16-133 block each other's binding to the receptor only marginally or not at all, because the determinant recognized by KJ1-26 is sensitive to reduction and alkylation unlike the target of KJ16-133, and because the binding of one antibody is temperature sensitive and the other not. Taken together, these data suggest that the most likely target of KJ16-133 binding is a determinant on one of the constant regions of the T cell receptor polypeptides. Gascoigne et al. (29), for example, have described two similar but not identical constant regions for one of the chains of the T cell receptor, both expressed on peripheral T cells. It is possible that KJ16-133 distinguishes between these. It should be pointed out, however, that if a constant region isotype is being defined by KJ16-133, this is not related to the specificity or function of the T cell itself since both Class II-reactive, helper T cell hybridomas (2, 9) and a Class I-reactive, cytotoxic T cell hybridoma (10) bear the determinant.

It is difficult to account for the temperature sensitivity of the binding of KJ16-133 to cells, since so little is known about the way in which the receptor operates, and how it is exposed at different temperatures. All biochemical and molecular



biological data point to the idea that at least one of the chains of the receptor is a transmembrane protein (1-6, 27, 28), since the molecule appears to be released from T cells bearing it only by detergent solubilization. Unpublished data from our laboratory have also shown that it may be stripped from cell surfaces by proteolysis. In man (30, 31), and perhaps in mouse (Dr. J. Allison, personal communication), it is also known that the receptor is closely associated with the complex of T3 proteins. Our current hypothesis is that a determinant on the receptor that lies close to the membrane or the T3 complex might be more readily or often exposed at 37°C because of increased membrane fluidity and that this accounts for the effects of temperature on KJ16-133 reactivity.

There is some question why the idiotypic determinant bound by KJ16-26 is so sensitive to reduction and alkylation, whereas that detected by KJ16-133 is not. Immunoglobulin molecules do not dissociate when reduced and alkylated at neutral pH, and  $V_H$  and  $V_C$  domains even remain associated in  $F_V$  fragments, suggesting that noncovalent, probably hydrophobic, forces hold the chains or fragments together (32). Whether or not reduced or alkylated immunoglobulins continue to react with anti-idiotypic reagents depends on the individual antibody (Dr. M. Geftter, personal communication). T cell receptors seem to be like immunoglobulins in that reduction and alkylation under nondenaturing conditions does not separate the two chains of the heterodimer, as shown by the continued precipitation of both chains by KJ16-133 with efficiency equal to precipitation of the nonreduced molecule. Lack of reactivity of this complex with KJ1-26 suggests that reduction does cause a change in configuration of the variable regions of the receptor.

A final point of interest about KJ16-133 is the strain distribution of its reactivity. The antibody reacts with BALB/c T cells ( $Igh^a$ ), and with T cells from most other strains tested, including several congenic with BALB/c at H-2 (BALB.K and BALB.B) and also B10.S(7R). It does not bind, however, T cells from SJL or SJA ( $Igh^b$ ) mice. These findings suggest that the genes controlling this determinant do not map to *Igh*, or to the MHC itself. A more extensive strain survey and mapping study will be published shortly.

### Summary

We have prepared a monoclonal antibody, KJ16-133, from the cells of a rat immunized with the purified receptor for antigen plus I-A of a BALB/c T cell hybridoma, DO-11.10. Unlike most other monoclonal anti-receptor antibodies that have been described before, KJ16-133 is not clone specific. It reacts with ~20% of the receptors on T cells of normal BALB/c mice. It also reacts with about the same percentage of antigen-specific, major histocompatibility complex (MHC)-restricted or allogeneic I-region specific T cell hybridomas. Reaction of KJ16-133 with a given T cell hybridoma does not seem to depend on the antigen specificity or MHC-restricting element of the T cell in question.

The determinant recognized by KJ16-133 has some unexpected properties. It is absent in several strains of mice including SJL/J and SJA/20, but present on the T cells of most other commonly used strains. The determinant recognized therefore does not map to *Igh*. Our experiments suggest that a clone-specific "anti-idiotypic" antibody and KJ16-133 recognize determinants on different parts

of the receptor. For example, the binding of a clone-specific antibody to target T cells is relatively temperature insensitive, whereas KJ16-133 binds well to cells at 37°C but poorly to cells at 4°C. The determinant recognized by a clone-specific antibody is sensitive to reduction and alkylation of the receptor, whereas KJ16-133 reactivity is not. Finally, binding of KJ16-133 at saturating concentrations to target T cells does not block the binding of a clone-specific antibody. Similarly, binding of a clone-specific antibody only marginally inhibits binding of KJ16-133.

Taken together, these results suggest that KJ16-133 is directed against an allelic determinant on T cells that may be close to the membrane, and not in the receptor binding site for antigen plus MHC. The antibody may recognize an allele of a constant region isotype, or an allele of a J region.

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