The T-cell accessory molecule CD4 recognizes a monomorphic determinant on isolated Ia

(planar membranes/T-cell activation)

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ABSTRACT The membrane protein CD4 is commonly found on mature T cells specific for antigen in association with class II major histocompatibility complex (MHC; Ia) proteins. This correlation has led to the suggestion that CD4 binds to a monomorphic region of the Ia molecule on the antigen-presenting cell (APC) and functions either by enhancing interaction between the T cell and the APC, or conversely, by transducing negative signals to the T cell. To address this hypothesis, we have made use of sublines from an unusual T hybrid that is class I MHC restricted but also CD4⁺. By incorporating purified MHC proteins into a planar membrane system, we show that different Ia molecules can greatly enhance the ability of a CD4⁺ but not a CD4⁻ variant of this class I-restricted T hybrid to respond to isolated class I molecules. T-cell responses can be strongly augmented by the concurrent expression of CD4 on the T cell and any of four different Ia proteins on planar membranes, thus supporting the idea that CD4 binds to a monomorphic region of the Ia molecule and increases the avidity with which the T cell can interact with its target.

T cells, by means of the T-cell receptor (TcR), recognize protein antigens presented in association with major histocompatibility complex (MHC) molecules on target/antigen-presenting cells (APCs) (1, 2). In addition to the TcR, mature T cells express "accessory" molecules—most notably either CD4 or CD8—on their cell surface membranes (3–6). The subpopulation of mature T cells expressing CD4 molecules consists mostly of T helper cells and, initially, CD4 was thought to define the T helper subset (3, 4). However, an even stricter correlation has been found between the accessory molecule expression of mature T cells and their MHC restriction (7, 8). Therefore, T cells expressing CD4 almost always recognize antigen presented in association with Ia (class II MHC) molecules. This finding has led to the idea that the function of CD4 is to interact specifically with Ia molecules on the APC, thereby strengthening the overall interaction between Ia-restricted T cells and APCs (9–12). Since no polymorphism for CD4 has been demonstrated, it follows that this molecule would have to recognize a conserved, monomorphic determinant on Ia. Ideally, the test of this hypothesis would rest on the demonstration of binding between purified Ia and purified CD4 molecules in solution. So far, however, evidence supporting this idea has come only from experiments in which interactions between intact cells have been measured (9–15). Here, in a first step toward the ideal studies, we used a planar membrane system containing purified Ia and purified TcR ligand to stimulate CD4⁺ and CD4⁻ versions of a T-cell hybridoma. The incorporation of each of four different Ia molecules into the planar membranes led to an enhanced stimulation of the CD4⁺ but not of the

CD4⁻ T-cell hybridoma. This enhanced response could be reduced to that caused by non-Ia-containing stimulatory planar membranes by the addition of anti-CD4 (GK-1.5) antibodies to the cultures. Thus, CD4 appears to facilitate T-cell activation by recognizing a monomorphic determinant on Ia.

MATERIALS AND METHODS

T Hybridomas Used. T-cell hybridomas were derived from the fusion of AKR thymoma BW5147 cells with T-cell blasts. The strain of origin and the immunizing antigen used to generate each T hybrid were as follows: 3DT-52.5 (and sublines), BALB/c, (TG)AL; DO-11.10, BALB/c, chicken ovalbumin; h4Ly-7.5, B10.A(4R), chicken egg lysozyme; 2B4, B10.A, pigeon cytochrome *c*; CG4, C57BL/6, H-2ᵈ. Sublines were produced by passaging the T-cell hybridoma 3DT-52.5 (characterized in refs. 13 and 16) for several months and then sorting for CD4 expression on an Ortho 50H cytofluorograph. Two consecutive sorts yielded the CD4⁻ subline 3DT-52.5/CD4⁻ and the CD4⁺ subline 3DT-52.5/CD4⁺. All cells were maintained in modified Mishell–Dutton medium containing 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, and 50 μg of gentamycin per ml (Shering, Kenilworth, NJ).

Cytofluorographic Analysis of T Hybrids. This technique has been described (14). Briefly, 10⁶ cells were incubated with either a murine anti-TcR idiotypic antibody directed against the TcR of 3DT-52.5 [KJ12-98 (1:50)] or the rat anti-mouse CD4 antibody GK-1.5 (1:200). Following several washes in phosphate-buffered saline (PBS)/2% fetal calf serum/0.5% sodium azide, a second incubation was carried out with either fluoroscein-coupled goat anti-mouse or goat anti-rat antibody. Cells were then washed and fixed with 0.2% paraformaldehyde and analyzed for fluorescence using a three-decade logarithmic scale with 256 channels on a Coulter EPICS V cytofluorograph.

MHC Protein Purification. IAᵏ, IEᵏ, IAᵈ, IEᵈ, and Dᵈ were isolated by means of affinity chromatography from Nonidet P-40 (NP-40) lysates of AKTb-1b (IAᵏ, IEᵏ), A20-1.11 (IAᵈ, IEᵈ), and P815 (Dᵈ) cells (17, 18). Typically, 5 × 10⁹–10¹⁰ cells were grown, either *in vitro* as spinner cultures (A20-1.11, P815) or *in vivo*, in the spleens of AKR mice (AKTb-1b). Cells were harvested, washed extensively with PBS (pH 7.2), and lysed with 1% NP-40/PBS. Lysates were then centrifuged to remove debris and passed over antibody-bound Sepharose 4B (Pharmacia) columns. IAᵏ protein was isolated by using the following antibody-coupled columns: 10-3.6 (anti-IAᵏ, IAᶠ, IAʳ, IAˢ), IAᵈ with MKD6 (anti-IAᵈ, IAᵖ, IAᵠ), IEᵈ and IEᵏ with 14-4.4 (anti-IE). Lysate passage was followed by extensive washing of the columns with 1% NP-40/PBS. Protein was then eluted with 50 mM diethyl-

amine/0.9% saline/1% octyl β-glucoside (pH range, 10.2 and 10.8 depending on the protein elution profile). Protein concentration of the eluant was determined by using the bicinchoninic acid assay (Pierce). One-hundred-microgram aliquots were stored at $-70°C$ until use. Purified protein was precipitated with 90% acetonitrile and analyzed by NaDodSO$_4$/PAGE according to the procedure of Laemmli (19).

Formation and Analysis of MHC Protein-Containing Planar Membranes. Protein incorporation into liposomes and subsequent fusion onto glass beads were performed as described (17, 20). Depending upon the competition between two proteins for incorporation into liposomes, the protein:lipid ratios varied as follows: (*i*) liposomes containing one protein: 14 μg of D^d, 20 μg of IA^d, or 25 μg of IA^k, IE^k, or IE^d to 200 μg of lipid (egg phosphatidylcholine:cholesterol, 7:2 M ratio); (*ii*) liposomes containing two proteins: 16 μg of D^d + 20 μg of IA^d, 16 μg of D^d + 25 μg of IA^k, 16 μg of D^d + 25 μg of IE^k, or 16 μg of D^d + 25 μg of IE^d to 400 μg of lipid. All mixtures were suspended in a final volume of 500 μl of PBS/1% octyl β-glucoside and dialyzed against PBS at 4°C for 24 hr. The resulting liposome-containing suspensions were mixed with a 1:1 (vol/vol) ratio of glass beads (diameter, 80 μm) for 24 hr and then washed once with PBS.

Enzyme-Linked Immunosorbent Assay (ELISA) of MHC Proteins on Planar Membranes. Relative concentrations of MHC molecules in liposomes bound to glass beads were determined by using an ELISA and monoclonal antibodies as described (10). Ten microliters of liposome-coated glass beads was incubated with an excess concentration of antibody at 20°C for 2 hr. The beads were then washed extensively with PBS and incubated with alkaline phosphatase-coupled anti-mouse immunoglobulin antibody for 2 hr at 4°C. The beads were washed again and incubated a final time with 60 mM *p*-nitrophenyl phosphate at 37°C. Color change was noted by optical density per well using an EAI reader (Bio-Tek, Burlington, VT) fitted with a 405-nm filter. ELISA readings of increasing quantities of a single protein on 10 μl of beads did not show a linear progression. Commonly, the lower protein concentrations gave slightly higher readings than anticipated.

Stimulation Assay. Ten microliters of liposome-bound beads was cocultured with 10^5 T-cell hybrids for 24 hr at 37°C in 96-well culture plates (Flow Laboratories) (final volume, 300 μl in culture medium). The rat anti-CD4 antibody GK-1.5 (final dilution, 1:100 ascites) was added at the beginning of the culture period. After 24 hr, the culture supernatants were assayed for the presence of interleukin 2 (IL-2) by their ability to support the growth of the IL-2-dependent T-cell line HT-2 (21). The highest of 1:2 serial dilutions capable of maintaining 90% HT-2 cell viability defined the IL-2 concentration of the supernatant. Ten units IL-2 per ml was the minimum concentration measurable.

Values obtained from numerous experiments show that the response by 3DT-52.5/CD4$^+$ to D^d-containing planar membranes on average increased by 6- to 13-fold when Ia molecules of any of the four species were also included in the planar membranes (data not shown).

Liposome-fused beads lacking MHC protein were incapable of stimulating any of the T-cell hybrids to IL-2 production.

RESULTS

Production and Analysis of T Hybrids. T helper cells are predominantly CD4$^+$ and Ia restricted. Because TcR and CD4 potentially recognize the same Ia molecule on the APC, it has been very difficult to assess the contribution of the CD4 molecule to T-cell recognition and/or activation. Following the example of Greenstein *et al.* (13), we reasoned that it would be helpful to employ a T cell bearing a TcR with a

specificity for MHC different from that predicted for the CD4 molecule. We therefore used an unusual murine T hybrid, 3DT-52.5 (characterized in refs. 13 and 16), which is CD4$^+$ but has a TcR that recognizes the murine class I molecule D^d. Blackman *et al.* (22) have established that the D^d-specific TcR on 3DT-52.5 is a hybrid molecule composed of the α-chain from the fusion partner BW5147 and the β-chain from an incoming T-cell blast. [Its presence on the cell surface of 3DT-52.5 can be detected with the anti-idiotypic antibody KJ12.98 (16)]. Although other receptors can potentially be formed, they are undetectable by cytofluorographic analysis (14).

To increase the relative contribution of the putative CD4/Ia interaction to the activation of 3DT-52.5, sublines that expressed suboptimal amounts of TcR were generated. Cytofluorographic analysis of these sublines indicated that 3DT-52.5/CD4$^-$ was a pure CD4$^-$ population with very low amounts of TcR (Fig. 1), and 3DT-52.5/CD4$^+$ was a population with slightly higher concentrations of TcR and a bimodal distribution of CD4. In all other respects, these lines appeared to be identical—i.e., cytofluorographic analysis indicated them to have identical expression of other surface markers such as Thy-1, class I MHC, and LFA-1, and they exhibited identical responses to MHC-unrestricted stimuli such as concanavalin A (in addition, other CD4$^+$ and CD4$^-$ variants have been derived from 3DT-52.5 with similar response patterns as those observed for 3DT-52.5/CD4$^+$ and 3DT-52.5/CD4$^-$, respectively).

Planar Membranes Containing MHC Proteins. The planar membrane system, as developed by Brian and McConnell (23) and later modified (17, 24), was used to present purified TcR ligand (D^d) and purified Ia to the T hybrids. This system had several advantages over the use of APCs in that (*i*) the use of highly purified protein preparations would limit the possible ligand candidates to only those introduced into the liposomes and (*ii*) the concentration of the TcR target ligand D^d could be maintained at a constant level in the presence or absence of other proteins. Therefore, a baseline stimulation could be elicited with planar membranes containing D^d alone and compared to that elicited by planar membranes containing D^d and Ia.
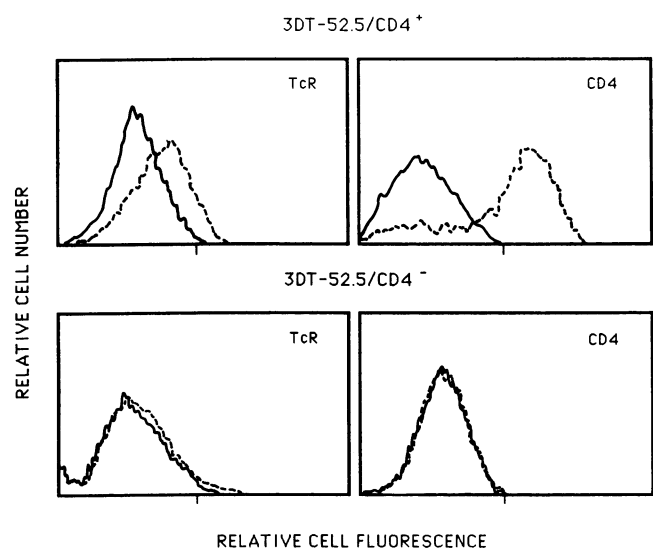

FIG. 1. Relative concentrations of TcR and CD4 on the T-cell hybridomas 3DT-52.5/CD4$^+$ and 3DT-52.5/CD4$^-$. Solid lines represent cells incubated with fluorescein isothiocyanate (FITC)-coupled secondary antibody alone. Dotted lines represent cells first stained either with anti-TcR antibody or with anti-CD4 antibody and then followed by incubation with FITC-coupled secondary antibody.
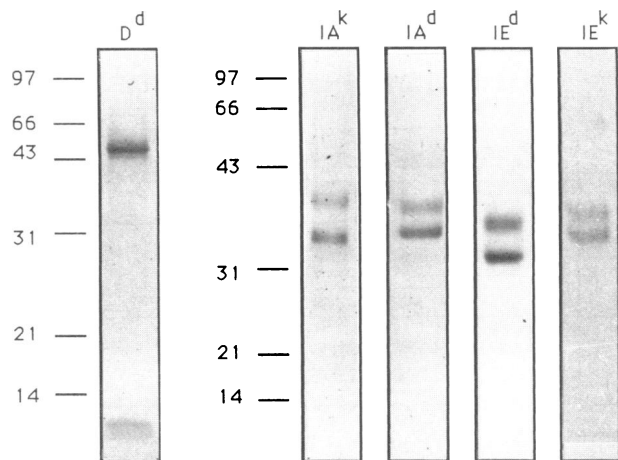
FIG. 2. NaDodSO$_4$/PAGE analysis of purified MHC proteins. The proteins were analyzed on 12% polyacrylamide gels, D^d and IE^d under nonreducing conditions and IA^k, IA^d, and IE^k under reducing conditions. Gels were stained with Coomassie blue. Molecular masses are indicated in kDa.

Fig. 2 shows a Coomassie blue-stained NaDodSO$_4$/PAGE analysis of the D^d and Ia preparations used in these planar membrane experiments. The D^d protein retained the β_2-microglobulin upon reprecipitation of purified D^d, suggesting that this molecule would also be in association with D^d in the planar membranes (data not shown). Purified protein was mixed with the appropriate lipids (phosphatidylcholine:cholesterol, 7:2 M ratio) and liposomes formed by detergent dialysis (17, 23, 24). The resulting liposomes were then fused onto clean glass beads.

To optimize the system, it was important to make the D^d concentration limiting while allowing for maximum incorporation of Ia. Stimulation of the 3DT-52.5 sublines could not be detected at an input D^d concentration below 25 μg/ml (data not shown). Therefore, a concentration of 25 μg/ml was commonly used in preparing liposomes. Addition of a second protein, such as Ia, slightly lowered the efficiency with which D^d was inserted into the liposomes. Therefore a higher initial D^d concentration was required in preparing liposomes containing both proteins. This ensured that D^d was incorporated into liposomes at equal concentrations in all preparations.

Table 1. ELISA determination of relative protein concentrations on planar membranes

MHC molecules incorporated	Relative ELISA readings*				
	D^d	IA^d	IA^k	IE^d	IE^k
D^d	1.00	0.07	0.10	0.07	0.05
D^d + IA^d	*0.95*	*1.04*	0.08	0.10	ND
IA^d	0.07	1.00	0.10	0.07	0.09
D^d + IA^k	*0.93*	0.07	*1.07*	0.15	ND
IA^k	0.11	0.05	1.00	0.16	0.01
D^d + IE^d	*0.96*	0.06	0.07	*1.08*	ND
IE^d	0.07	0.04	0.08	1.00	0.06
D^d + IE^k	*1.00*	ND	ND	ND	*0.94*
IE^k	0.02	0.02	0.05	ND	1.00

ND, not determined.
*The values obtained using a given monoclonal antibody (D^d, IA^d, IA^k, IE^d, IE^k) were normalized to the reading obtained with the planar membranes containing only the appropriate protein. Numbers in italics indicate values for proteins deliberately incorporated into the membranes.

ELISA results shown in Table 1 indicate that all sets of planar membranes containing D^d had approximately equal concentrations of D^d. All Ia proteins appeared to incorporate into liposomes containing only that protein or both proteins with equal efficiency.

Planar Membrane Stimulation of Class II-Restricted T Hybridomas. The planar membranes were first tested for their capacity to stimulate Ia-restricted T hybrids. Membranes containing IA^k, IA^d, or IE^k presented predigested antigen in an Ia-restricted fashion, and IE^d-containing membranes stimulated an alloreactive T hybrid (CG4) in the absence of exogenous antigen (Fig. 3). Addition of D^d to these planar membranes affected the Ia-restricted stimulation marginally, if at all. A slight decrease in response was sometimes observed with addition of D^d, indicating that its presence might sterically hinder the ability of the TcR to bind to class II molecules (this phenomenon was observed in $\approx$50% of the experiments and was not consistently observed for any of the T hybrids). Neither anti-LFA-1 nor anti-class I antibodies affected stimulation in the presence or absence of D^d (data not shown). Thus, the coincorporation of D^d into Ia-containing planar membranes did not enhance Ia-restricted responses by CD4$^+$ T-cell hybridomas.
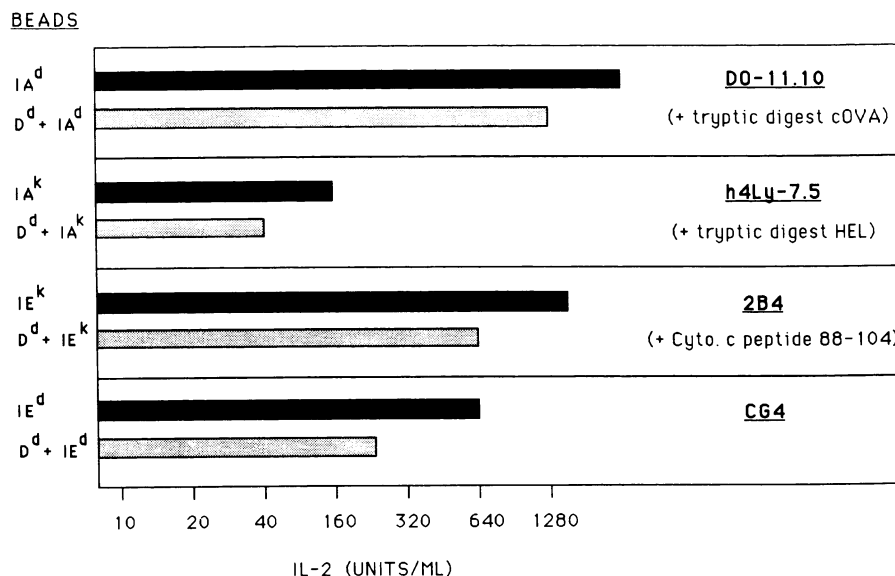

FIG. 3. Addition of D^d protein to Ia-containing planar membranes does not augment response of class II-restricted T hybrids. Stimulation of T hybrids was gauged by IL-2 secretion.
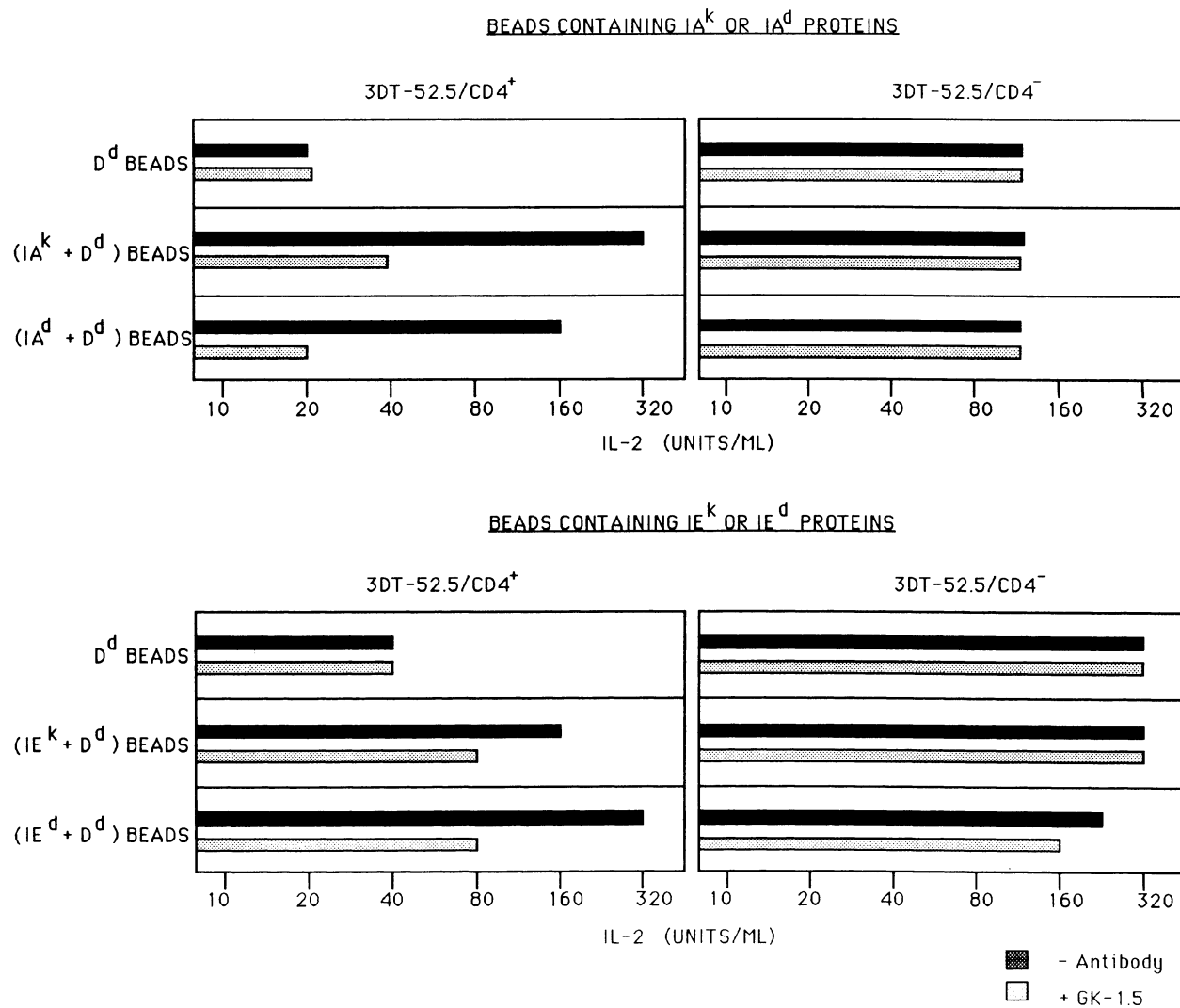
BEADS CONTAINING IA^k OR IA^d PROTEINS

BEADS CONTAINING IE^k OR IE^d PROTEINS

FIG. 4.   Addition of Ia proteins to D^d planar membranes greatly enhances stimulation of $CD4^+$ but not $CD4^-$ class I-restricted T hybrids. The assay was as for Fig. 3.

Planar Membrane Stimulation of Class I MHC-Restricted T Hybrids. Fig. 4 shows the results of a typical experiment examining the capacity of planar membranes containing D^d and Ia molecules to stimulate 3DT-52.5/$CD4^+$ and 3DT-52.5/$CD4^-$. Both variants responded to planar membranes containing D^d alone, again showing that this molecule is the ligand of the 3DT-52.5 TcR (22). As expected, neither of the two 3DT-52.5 sublines was stimulated by IA^d, IA^k, IE^k, or IE^d in the absence of D^d (data not shown). Incorporation of IA^k, IA^d, IE^k, or IE^d into the D^d-containing membranes did not affect the response of 3DT-52.5/$CD4^-$ and, as expected, anti-CD4 antibodies had no effect on the stimulation of this hybrid. In contrast, 3DT-52.5/$CD4^+$ responded much better to D^d when any of the Ia species (IA^d, IA^k, IE^k, or IE^d) was also incorporated into the D^d-containing planar membranes, and the addition of anti-CD4 antibodies reduced this response to the levels elicited by planar membranes containing D^d alone. Anti-Ia antibodies proved to be poor inhibitors of this response (data not shown); however, all those used were murine and presumably not directed against the highly conserved CD4-binding site.

DISCUSSION

We have used planar membranes containing the class I MHC protein D^d with or without the addition of class II MHC Ia proteins to stimulate $CD4^+$ and $CD4^-$ D^d-reactive T hybrid-

omas. Results show that a substantial increase in response over that induced with D^d alone could be elicited when CD4 was expressed by the T hybrid and Ia was present on the D^d-containing planar membranes. The fact that this increase absolutely required the presence of CD4 on the 3DT-52.5 T-cell hybridoma as well as Ia on the D^d membranes indicates that CD4 interacts with Ia leading to an enhanced interaction between T cells and APC. That all four Ias tested were able to facilitate this enhancement indicates that CD4 recognizes a conserved monomorphic determinant on Ia.

The response to D^d by 3DT-52.5/$CD4^+$ in the absence of Ia was not affected by the presence of anti-CD4 antibodies (Fig. 4). This result is consistent with our previous results (17) but contrasts with the findings of others that showed anti-CD4 antibodies could inhibit marginal T-cell activation in the absence of Ia, suggesting that CD4 functions as a negative signaling molecule (25, 26). The discrepancy between our results and the findings of others might suggest differences in the initial activation level of T hybrids as compared with that of normal T cells. Thus "marginal" stimulation of the T hybrids might be more difficult to elicit.

Anti-LFA-1 antibody had no effect on stimulation of either hybrid (data not shown), presumably because the LFA-1 target ligand was not included in the planar membranes. In contrast, addition of anti-D^d antibody completely abrogated response by either $CD4^+$ or $CD4^-$ hybrid to any of the D^d-containing planar membranes (data not shown). Surpris-

ingly, a number of broad-specificity anti-Ia monoclonal antibodies failed to inhibit augmented response of the CD4$^+$ T hybrids to D^d + Ia planar membranes. This finding, which is in contradiction to the work of Greenstein *et al.* (13), might reflect structural variations in Ia protein inserted into cell membranes as opposed to planar membranes that could reduce the binding affinity of certain antibodies. However, this did not appear to be the case because all of those tested for inhibition appeared to bind equally well to Ia$^+$ cells and planar membranes (data not shown). A more likely explanation for this finding was that the antibodies used in our studies were not directed against the monomorphic CD4-binding region. It should be noted that although these murine antibodies exhibited broad specificity, none recognized Ia epitopes common to all haplotypes.

Although considerable homology exists between class I and class II MHC molecules, the CD4-binding site does not appear to reside on class I protein (at least, not D^d) because, as mentioned above, inhibition of response with anti-CD4 antibodies, clearly observed when any of the four Ias was included in the D^d planar membranes, was not seen when D^d alone was in the planar membranes (Fig. 4). Also, addition of D^d to Ia$^+$ planar membranes did not augment the response of class II MHC-restricted T hybrids, even with suboptimal concentrations of antigen and Ia (Fig. 3). Thus, the CD4 ligand appears to be a monomorphic determinant present only on class II MHC proteins.

It is evident from data presented here and elsewhere (13, 14) that T-cell stimulation does not require recognition of the same MHC molecule by TcR and CD4. Nevertheless, evidence indicates that the TcR and CD4 molecules lie in close proximity to one another on the T-cell membrane and may typically bind to the same Ia molecule (27). Weyland *et al.* (28) have shown that mitogen-induced T-cell activation causes capping of TcR and CD4, and Saizawa *et al.* (29) have recently demonstrated that antibody-induced capping of TcR results in the comodulation of CD4. In addition, by fluorescence microscopy we have observed that CD4 capping by anti-CD4 antibodies induces cocapping of TcR and CD4 to the same location on the T cell (unpublished results). Finally, data in this paper show that the CD4$^-$ hybrid 3DT-52.5/CD4$^-$ responded better to D^d in the absence of Ia than did its CD4$^+$ counterpart (Fig. 4), even though 3DT-52.5/CD4$^-$ had lower TcR concentrations than did 3DT-52.5/CD4$^+$ (Fig. 1). This indicates that even though CD4 greatly aids 3DT-52.5/CD4$^+$ in the presence of Ia, it may interfere with TcR function, perhaps sterically, in the absence of Ia. This finding also suggests that CD4 may lie in close proximity to the TcR on the cell surface and that both molecules may typically bind to the same MHC molecule on the APC.

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