THE MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED ANTIGEN RECEPTOR ON T CELLS. II. ROLE OF THE L3T4 PRODUCT*

By PHILIPPA MARRACK, ROBERT ENDRES, RICHARD SHIMONKEVITZ, ALBERT ZLOTNIK, DENO DIALYNAS, FRANK FITCH, AND JOHN KAPPLER‡

From the Department of Medicine, National Jewish Hospital and Research Center, Denver, Colorado 80206; and Departments of Microbiology and Immunology and Biophysics, Biochemistry and Genetics and Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262; and Departments of Microbiology and Pathology, University of Chicago, Chicago, Illinois 60637

A number of recent publications have suggested that the antigen called T4 or Leu-3 in man or L3T4 in mouse is intimately involved in the recognition of antigen (Ag)[1] in association with Class II products of the major histocompatibility complexes (MHC) of the respective species. Thus T4 monoclonal antibodies block T cell proliferation in response to Class II alloantigens in man, and also block killing mediated by recognition of Class II molecules (1–7). Similar findings have been obtained in mouse (8, 9). In these respects the Leu-3, T4 or L3T4 molecule seems to serve a function for Class II-restricted T cells analogous to the function of Leu-2 or T8 molecules in man or Lyt-2 molecules in mouse for Class I-restricted T cells (10–16).

These results have been used by several authors to support theories which propose that these apparently nonvariant molecules act in recognition of antigen by T cells by binding to some nonpolymorphic molecule, or perhaps some nonpolymorphic region of Class I or Class II molecules. By so binding they are presumed to increase the overall avidity of the T cell for its target cell (3, 5, 16, 17). Thus T cell binding to Ag presenting cells is presumed to be mediated by clone-specific receptors on the T cell for Ag/MHC, and also by the T4, L3T4 or T8, Lyt-2 molecules. Distribution studies, and perhaps common sense, predict that T4, L3T4 would be present on and enhance binding of Class II–restricted T cells.

Other interpretations of the data are possible, however. For example, these nonpolymorphic molecules might serve an anchoring, or signaling, function for

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[1] *Abbreviations used in this paper:* Ag, antigen; Con A, concanavalin A; FBS, fetal bovine serum; IL-2, interleukin-2; MHC, major histocompatibility complex; ND, not done; OD, optical density; Oua, ouabain; OVA, ovalbumin; PBS, phosphate-buffered saline; SE, standard error; SN, supernatant; (TG)AL, poly-L(Tyr,Glu)-poly-D,L-Ala—poly-L-Lys.

the Ag/MHC receptor on T cells.

In this paper we present data which suggest that the former interpretation is correct. Thus we have examined a collection of Ag/I-specific murine T cell hybridomas for the presence of the L3T4 antigen. All the primary clones bore this antigen. Recognition of Ag/I by these T cell hybridomas was, however, variably inhibited by monoclonal antibody to L3T4. This inhibition appeared to correlate with the overall sensitivity of the T cell hybridoma to Ag/I. Thus, hybrids that reacted well with low doses of Ag, or were difficult to inhibit with anti-I reagents, i.e. that appeared to be relatively sensitive to Ag/I, were also difficult to inhibit with anti-L3T4. This suggested that the L3T4 molecule acted to increase the sensitivity of T cells to Ag/I, rather than act in some essential fashion in Ag/I recognition.

This conclusion was substantiated by our discovery that three subclones of one of our T cell hybridomas, D0-11.10, had lost the L3T4 antigen but retained comparable quantities of the Ag/I receptor on their surfaces. The $L3T4^-$ subclones had lower sensitivity to Ag/I, since they responded less well to challenge with Ag/I, and were more easily inhibited by anti-I antibodies than their parent.

Materials and Methods

Culture Medium. Monoclonal antibody-producing B cell hybridomas, B cell lymphomas, and T cell hybridomas were all cultured in medium prepared by the recipe of Mishell and Dutton (18) with the exception that the medium was fed before use with 10% nutrient cocktail (18) and medium contained 10% fetal bovine serum (FBS), 5×10^5 M 2-mercaptoethanol, and 50 μg/ml gentamycin (Schering Corp., Kenilworth, NJ).

Reagents. Chicken ovalbumin (OVA) from Sigma Chemical Co., St. Louis, MO, and poly-L(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [(TG)AL] from Miles Laboratories, Inc., Elkhart, IN, were used as Ag in these experiments. Concanavalin A (Con A), alkaline phosphatase–conjugated rabbit anti-mouse IgG and para-nitrophenyl phosphate were also purchased from the Sigma Chemical Co.

Monoclonal Antibodies. Monoclonal antibodies were prepared either as the cell-free supernatant (SN) of hybridoma cultures grown to $\sim 10^6$ cells/ml or as the ascitic fluid of irradiated mice carrying the hybridoma tumor. A list of the monoclonal antibodies, and their sources, used in these experiments is shown in Table I.

Monoclonal antibodies prepared as culture supernatants were used neat in our experiments. KJ1-26.1 ascitic fluid was used at a final concentration of 1:250. Concentrations of GK1.5, MK-D6, and M5/114 varied according to experimental protocols as indicated

TABLE I

Monoclonal Antibodies Used in These Experiments

Monoclonal antibody	Target molecule	Source	Reference
GK1.5	Murine L3T4	Culture SN	Dialynas et al. (8)
KJ1-26.1	Ag/I receptor on T cell hybridoma DO11.10	Ascites	Haskins et al. (19)
T24/31.7	Thy-1	Culture SN	Dennert et al. (20)
1/2117.7	LFA-1	Culture SN	Trowbridge et al. (21)
FD-441.8	LFA-1	Culture SN	Sarmiento et al. (22)
FD-18.5	LFA-1	Culture SN	Sarmiento et al. (22)
MK-D6	$I\text{-}A^d$	Ascites	Kappler et al. (23)
M5/114	$I\text{-}A^{d,b}$, $I\text{-}E^{d,k}$	Culture SN	Bhattacharya et al. (24)

in the Results section.

T Cell Hybridomas. T cell hybridomas for these experiments were prepared by fusion of Ag/MHC-reactive T cell blasts to BW5147 by standard procedures (23). The properties of many of the T cell hybridomas used have been described elsewhere (19, 25, 26); all are listed in Table II. For the experiments described in this paper we chose a panel of hybridomas, almost all of which were restricted by a single MHC product, I-A^d. This enabled us to compare the reactions of these T cells more accurately.

Subclones of the T cell hybridoma D0-11.10 were prepared by passaging the cloned hybridomas in tissue culture for 8–16 wk and then recloning the hybrids at limiting dilution. In one case the cells had been selected for resistance to 8-azaguanine and to ouabain (Oua) before cloning. Subclones were assayed for their ability to respond to the appropriate Ag/MHC or, as a control, Con A, by production of interleukin-2 (IL-2).

IL-2 Assay for Ag/MHC Recognition by T Cell Hybridomas. Recognition of Ag/MHC by T cell hybridomas was assayed by the secretion of IL-2 by these hybrids as previously described (23). None of these hybridomas made detectable IL-2 in the absence of the appropriate Ag/MHC. To induce IL-2 production 5×10^4 hybridoma T cells were incubated for 24 h with the appropriate Ag and 10^5 Ag-presenting cells in 250-μl cultures. Except as noted, antigen was added at a final concentration of 500–1,000 μg/ml. In most cases the H-2^d-bearing B cell lymphoma A20-2J or a close relative, A20-1.11 were used as Ag-presenting cells (27). Both these cell lines bear and present all expected H-2 products including I region products (28).

After 24 h SNs of these cultures were titrated in serial twofold dilutions and assayed for concentration of IL-2 by their ability to support the growth of the IL-2–dependent T cell line, HT-2. The highest dilution of culture SN able to maintain 4,000 HT-2 cells at 90% viability after 24 h culture was said to contain 1 unit of IL-2. Results in this paper are expressed as U/ml IL-2 secreted by the T cell hybridomas under different conditions. 10/ml IL-2 was the minimum detectable using this assay.

ELISA for Surface Antigens on T Cell Hybridomas. T cell hybridomas were assayed for surface antigens using ELISA. The inner 60 wells of 96-well microculture plates were precoated with 30 μl FBS. Antibodies were then added to these wells in 100-μl aliquots at saturating concentrations. T cell hybridomas to be assayed were then added at 3×10^5 cells/well in 30 μl culture medium. The outer wells of the 96-well culture plates were filled with balanced salt solution. The plates were placed in plastic bags, gassed with a 90% air, 10% CO_2 mixture, and incubated on ice for 1 h. The SNs were removed and the plates washed three times with 175 μl/well phosphate-buffered saline (PBS), 2% FBS, centrifuging between washes to pellet the cells. A 1:500 dilution of alkaline phosphatase-coupled rabbit anti-mouse IgG in PBS, 2% FBS was then added in 100-μl aliquots to assay wells, the cells were resuspended in this mixture, and incubated at room temperature for

TABLE II

T Cell Hybridomas Used in These Experiments

T cell hybridomas	Ag priming used to generate normal T cell parent*	T hybridoma specificity
DO-11.10	OVA	OVA/I-A^d
3DT-18.11	(TG)AL	(TG)AL/I-A^d
3DT-52.5	(TG)AL	D^d
3DO-8.2	OVA	OVA/I-A^d
3DO-18.3	OVA	OVA/I-A^d
3DO-20.10	OVA	OVA/I-A^d
3DO-54.8	OVA	OVA/I-A^d
4DO-44.1	OVA	I-A^d

*All hybrids were generated by fusion of BALB/c T cell blasts to the T cell thymoma, BW5147. The properties of these T cell hybridomas are described in greater detail in references 19, 25, and 26.

1 h. At this point SN were again discarded, and the cells washed five times with 175 μl/well aliquots of PBS, 2% FBS. Substrate, para-nitrophenyl phosphate, was then added to assay wells in 175-μl aliquots as a 1.67 mg/ml solution in 0.1 M glycine buffer, pH 10.4, containing 1 mM magnesium chloride and 1 mM zinc chloride. The plates were incubated for a further hour at 37°C before reading optical density (OD) with an ELISA reader using a 405-nm filter (Bio-Tek Instruments, Inc., Burlington, VT). Results are expressed as the average OD ± standard error (SE) after subtraction of the background OD of cells tested in the absence of the initial test, antibody. Assay cultures were always performed in triplicate, SE was calculated by appropriate combinations of the SE of experimental and background cultures.

Results

Expression of the L3T4 Molecule by T Cell Hybridomas. We measured the relative amounts of L3T4 antigen on different Ag/MHC-specific T cell hybridomas using ELISA. As control Ag we also measured the presence of Thy-1, LFA-1 and, in the case of D0-11.10, receptor for OVA/I-A^d using a monoclonal antibody we have recently prepared, KJ1-26.1, which reacts with this receptor (19). The results of several such assays are shown in Table III.

Three different experiments are listed in Table III. Readings for the T cell hybridoma D0-11.10 are included for each experiment as a "constant" cell line with which values for other T cell hybridomas can be compared. As shown, all the Ag-specific MHC-restricted T cell hybridomas tested bore L3T4, Thy-1, and LFA-1, albeit in variable amounts. The parent tumor for these T cell hybridomas, BW5147, did not bear the L3T4 antigen. Surprisingly, we found the L3T4 antigen on 3DT52.5. This T cell hybridoma was isolated after fusing T cell blasts, putatively specific for (TG)AL/H-2^d, to BW5147. 3DT52.5, however, responds to D^d, as indicated by mapping and, more recently, transfection experiments (reference 26, and Dr. Julia Greenstein, personal communication). Pre-

TABLE III

Expression of L3T4 and Other Surface Antigens by T Cell Hybridomas

Exp.	T cell hybridoma	OD ± SE (× 10^3) using antibodies to:			
		L3T4*	Thy-1‡	LFA-1§	DO-11.10^d OVA/I-A^d receptor
I	DO-11.10	143 ± 23	292 ± 8	287 ± 3	310 ± 28
	3DT-18.11	61 ± 4	245 ± 7	229 ± 3	9 ± 4
	3DT-52.5	102 ± 5	200 ± 8	266 ± 5	0 ± 3
II	DO-11.10	39 ± 6	116 ± 5	119 ± 5	ND
	3DO-8.2	19 ± 6	144 ± 5	79 ± 5	ND
	3DO-18.3	26 ± 6	127 ± 7	100 ± 4	ND
	3DO-20.10	26 ± 6	185 ± 5	118 ± 6	ND
	3DO-54.8	67 ± 11	101 ± 23	57 ± 10	ND
	4DO-44.1	100 ± 10	135 ± 24	83 ± 17	ND
III	DO-11.10	95 ± 3	188 ± 5	ND	301 ± 7
	BW5147	5 ± 4	337 ± 7	ND	11 ± 5

* Supernatant of GK1.5 (8).
‡ Supernatant of T24/31.7 (20).
§ Supernatant of FD-441.8 or corrected reading using SN of FD-18.5 or I/2117.7 (21, 22).
‖ Ascites 1:250 dilution of KJ1-26.1 (19).
ND, not done.

vious experience would suggest that a Class I-specific hybridoma should bear Lyt-2, the T8 homologue, not the murine homologue of L3T4. We have used ELISA methods to check for the presence of Lyt-2 on 3DT52.2, and been unable to find the antigen. All our T cell hybridomas are Lyt-2$^-$ by such an assay, however, thus in the absence of a positive control we cannot conclude absolutely that 3DT52.5 is Lyt-2$^-$, only that it is L3T4$^+$. Our data do suggest this, though.

Effect of Anti-L3T4 on Ag/MHC Recognition by T Cell Hybridomas. Our previous studies and those of others have shown that the responses of many Class II-specific T cells are very well inhibited by anti-Leu-3 or T4 in man or anti-L3T4 in mouse (1–8). By contrast we have found that recognition of Ag plus Class II molecules by T cell hybridomas is variably inhibited by anti-L3T4 (9). This point is further illustrated in Table IV.

Various T cell hybridomas were incubated with appropriate Ag/MHC in the presence of different concentrations of anti-L3T4, and recognition of Ag/MHC monitored by production of IL-2. As shown in Table IV, some T cell hybridomas, for example 3DT-18.11 or 3D0-20.10, were very effectively inhibited by the antibody, whereas others, most notably 3D0-18.3 or 3D0-54.8, were unaffected, or only inhibited at high concentrations of anti-L3T4. From these data we could establish an approximate rank order of inhibition of these hybridomas as follows: – 3D0-20.10 > 3DT-18.11 > 4D0-44.1 > 3D0-8.2 > D0-11.10 > 3D0-54.8 > 3DT52.5 > 3D018.3. There was no correlation between the degree of inhibition of Ag/MHC recognition for a particular T cell hybridoma, and the amount of L3T4 detected on that hybrid by ELISA (Table III).

Degree of Inhibition with Anti-L3T4 is Related to the Affinity for Ag/MHC. It has been suggested before by us and others, that the Leu-3, T4 or L3T4 antigen contributes to Ag/MHC recognition by binding to some constant target, perhaps a constant portion of Class II proteins, on the target cell, thereby increasing the overall avidity of the T cell (3, 5, 8, 9, 17). Such a hypothesis is a direct analogue of the proposed role of Lyt-2 or T8 molecules in Class I recognition (16). This suggestion could certainly be used to explain the results shown above in Table IV, and if true, would predict that T cell hybridomas more easily inhibited by L3T4 would have a lower avidity for Ag/MHC. Since many of the T cell hybrids

TABLE IV

Inhibition of Ag/MHC Recognition by Anti-L3T4 Antibodies

T cell hybridoma*	U/ml IL-2 produced in response to Ag/MHC with anti-L3T4 (μl/culture)				
	5	1	0.2	0.04	0
3DO-18.3	2,560	2,560	2,560	2,560	2,560
3DT-52.5	80	160	160	320	320
3DO-54.8	80	160	1,280	2,560	1,280
3DO-8.2	20	40	40	320	320
DO-11.10	10	40	640	1,280	1,280
4DO-44.1	<10	<10	160	640	1,280
3DT-18.11	<10	<10	<10	160	640
3DO-20.10	<10	<10	<10	10	160

* Cultures contained 10^5 A20-1.11 Ag/I presenting cells plus 500 μg/ml Ag where required.

shown in Table IV are specific for Ag (OVA) plus I-A^d we decided to compare their avidities for Ag/I-A^d as well as we could by measuring the sensitivities of the T cell hybridomas to low Ag concentrations, and the ability of the T cell hybrids to recognize Ag/I-A^d in the presence of anti-I-A^d antibodies. High avidity T cell hybrids should be most able to react in the presence of low concentrations of Ag, and be least easily inhibited by anti-I-A^d antibodies.

As shown in Table V, different T cell hybridomas had different abilities to respond to low concentrations of Ag. Although these reactivities may reflect a number of factors, for example, the affinity of the T cell receptor for Ag/I and the ability of the Ag-presenting cells to process and present the appropriate Ag derivative, the approximate overall ordering of the avidity of the interaction between the T cell hybridomas and Ag/I^d as measured by these experiments was 3D0-20.10 < 3D0-8.2 < 3DT-18.11 < D0-11.10 < 3D0-54.8 < 3D0-18.3.

A similar rank order for the overall avidity can be derived from the results shown in Table VI, in which the response of T cell hybridomas to Ag/I-A^d was measured in the presence of different concentrations of the anti-I-A^d monoclonal antibody, M5/114 (24). In this case the approximate order was: 3D0-20.10 ≤ 4D0-44.1 ≤ 3D0-8.2 < 3DT-18.11 < D0-11.10 < 3D0-54.8 < 3D0-18.3.

Although these two orderings were not absolutely identical to that found with

TABLE V

Ability of T Cell Hybridomas to Recognize Different Concentrations of Ag

T cell hybridomas	U/ml IL-2 produced in response to Ag at:*				
	0	10	100	250	1,000
3DO-18.3	<10	320	1,280	1,280	1,280
3DO-54.8	<10	40	640	640	1,280
DO-11.10	<10	<10	320	640	>640
3DT-18.11	<10	<10	20	320	640
3DO-8.2	<10	<10	20	160	640
3DO-20.10	<10	<10	10	20	640

* Cultures contained 10^5 A20-1.11 Ag/I presenting cells plus Ag at the indicated concentration in μg/ml.

TABLE VI

Ability of Different Concentrations of Anti-I-A^d to Inhibit Ag/I-A^d Recognition by T Cell Hybridomas

T cell hybridoma	U/ml IL-2 produced in response to Ag/I-A^d with anti-I-A^d (μl SN/culture)*				
	50	10	2	0.4	0
3DO-18.3	10	1,280	>1,280	>1,280	>1,280
3DO-54.8	10	80	1,280	1,280	1,280
DO-11.10	<10	160	1,280	>1,280	1,280
3DT-18.11	<10	10	160	320	ND
3DO-8.2	<10	<10	80	320	320
4DO-44.1	<10	10	80	640	1,280
3DO-20.10	<10	<10	80	160	160

* Cultures contained M5/114 (24) at the indicated concentrations.
SN, supernatant.

anti-L3T4 inhibition, they were very similar, i.e. T cell hybridomas which appeared to interact with low sensitivity to Ag/I-A^d were more easily inhibited by anti-L3T4 antibody than T cell hybridomas with high sensitivity.

The hypothesis noted above for the way in which the L3T4 antigen works would also suggest that in the presence of anti-L3T4 antibodies, the avidity of T cell hybridomas for Ag/MHC on Ag-presenting cells should be lowered, and therefore the reaction between the two cells should be more easily inhibited by lowering Ag concentration, or antibodies to the relevant MHC product. We therefore titrated the effects of the monoclonal anti-I-A^d antibody, MKD6, on Ag/I-A^d recognition by several high sensitivity T cell hybridomas in the presence or absence of anti-L3T4 antibodies. Some representative results are shown in Fig. 1.

As discussed above, anti-L3T4, even at 1:250 dilution, clearly had some inhibitory effects on OVA/I-A^d recognition by the T cell hybridomas. In addition, the antibody synergized with anti-I-A^d in blocking responses. For example, for D0-11.10, anti-I-A^d at 1:1,250 reduced control responses from 1,280 to 640 U/ml IL-2, i.e. effective recognition was halved. By comparison, in the presence of limiting anti-L3T4, anti-I-A^d at 1:250 reduced responses from 640 to 10 U/ml IL-2, effective recognition was reduced 64-fold.

Properties of T Cell Hybridomas That Lack L3T4. T cell hybridomas that lacked L3T4 were produced (accidentally) during subcloning of several cloned T cell hybridomas. The properties of some of these clones are shown in Table VII.

D0-11.10, and all the subclones of this hybridoma shown, continued to bear approximately similar amounts of material that reacts with a monoclonal antibody against the OVA/I-A^d receptor on this cell (19). These clones also bore LFA-1 and Thy-1. Three of the subclones had lost the L3T4 antigen, and with this, their response to OVA/I-A^d was reduced, although it was not absent.

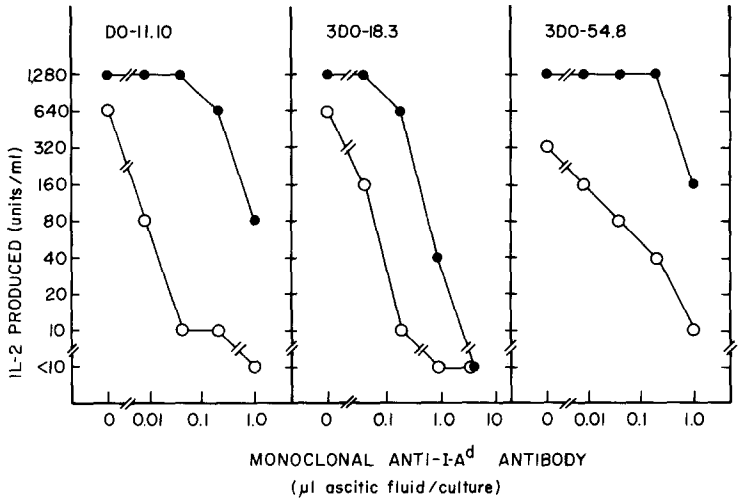

FIGURE 1. Anti-L3T4 lowers the overall avidity of T cells for Ag/MHC on Ag-presenting cells. The response of three T cell hybridomas to OVA/I-A^d was measured in the presence of various concentrations of the anti-I-A^d monoclonal antibody, MK-D6, in the absence (●) or presence (○) of a 1:250 dilution of anti-L3T4. Response was measured as U/ml IL-2 secreted.

Subclones of D0-11.10 that had lost L3T4 but retained receptors for OVA/I-A^d had several interesting properties. As shown in Table VII, they responded less well than the parent hybridoma to OVA/I-A^d presented, in this case, on A20-1.11 B cell lymphoma cells. They also responded less well, but detectably, to OVA presented by a number of other B cell lymphomas and in the case of D0-11.10.AG8.Oua1, by irradiated BALB/c spleen cells (data not shown). As shown in Table VIII, not unexpectedly, the response to OVA/I-A^d of the one L3T4$^-$ D0-11.10 subclone tested, D0-11.10.AG8.Oua1, was not inhibited by anti-L3T4 antibodies.

We reasoned that if the theory that the L3T4 molecule contributed to the overall avidity with which T cells react with their target cells was correct, then L3T4$^-$ hybrids bearing the same receptor for Ag/MHC in apparently the same amounts as an L3T4$^+$ hybridoma should have a reduced avidity for Ag/MHC-presenting cells. This is illustrated by the results shown in Figs. 2 and 3. The data in Fig. 2 show once again that the L3T4$^+$ hybridoma, D0-11.10, responded to OVA/I-A^d better than did its L3T4$^-$ subclone, D0-11.10.AG8.Oua1. Also, the parental clone continued to respond at lower doses of OVA than D0-11.10.AG8.Oua1, suggesting that the L3T4$^+$ clone had greater overall avidity for Ag/I on Ag-presenting cells than its L3T4$^-$ derivative. Fig. 3 shows results obtained when the response to OVA/I-A^d of these hybridomas was measured in the presence of different concentrations of the anti-I-A^d monoclonal antibody, MKD6. OVA/I-A^d recognition by the L3T4$^-$ subclone was more easily blocked

TABLE VII

Properties of T Cell Hybridomas Lacking L3T4

T cell hybridomas	OD ± SE (× 10^3) using antibodies to:				U/ml IL-2 produced in response to:	
	L3T4*	Thy-1*	LFA-1‡	DO-11.10* OVA/I-A^d receptor	Ag/I	Con A
DO-11.10	38 ± 4	203 ± 8	37 ± 3	191 ± 8	2,560	640
DO-11.10.24	75 ± 10	264 ± 3	67 ± 2	171 ± 4	2,560	640
DO-11.10.24.1	−1 ± 6	203 ± 7	44 ± 6	189 ± 12	80	80
DO-11.10.24.3	−2 ± 6	285 ± 31	47 ± 8	233 ± 10	40	160
DO-11.10.AG8.Ou.1	4 ± 4	218 ± 22	57 ± 5	338 ± 16	640	640

* See footnote to Table III.
‡ Supernatant of FD-18.5 (22).

TABLE VIII

OVA/I-A^d Recognition by an L3T4$^-$ T Cell Hybridoma Is Not Affected by Antibodies to L3T4

T cell hybridoma	U/ml IL-2 produced in response to OVA/I-A^d in the presence of anti-L3T4 (μl SN/culture)				
	5	1	0.2	0.04	0
DO-11.10	10	40	640	1,280	1,280
DO-11.10.AG8.Oual	80	80	80	80	40

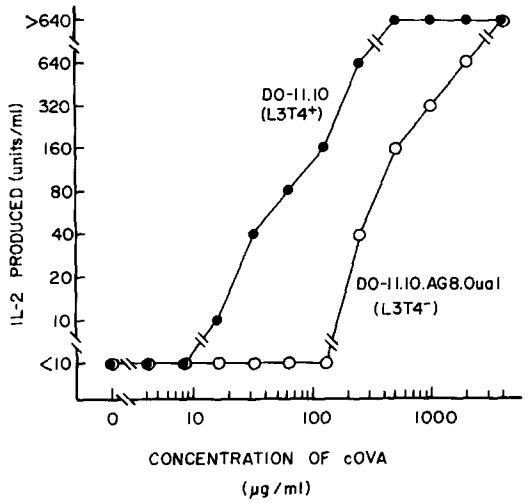

FIGURE 2. Lack of L3T4 renders T cell hybridomas less sensitive to low doses of Ag. The $L3T4^+$ T cell hybridoma, D0-11.10 (●) and its $L3T4^-$ subclone, D0-11.10.AG8.Oua1 (○) were challenged with Ag-presenting cells and different concentrations of OVA. Response was measured as U/ml IL-2 secreted.

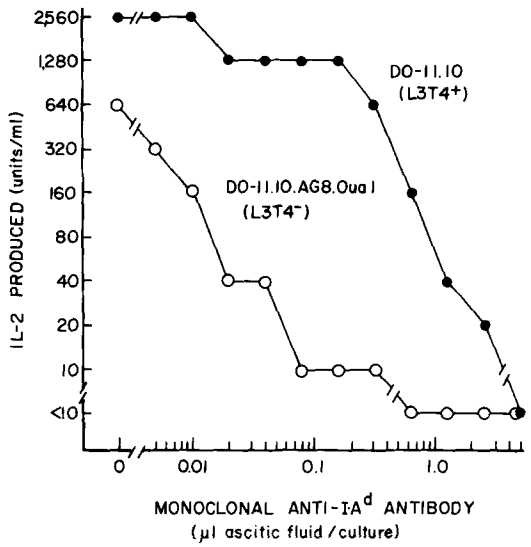

FIGURE 3. Lack of L3T4 renders T cell hybridomas more easily inhibitable by anti-I-A^d. The OVA/I-A^d-specific $L3T4^+$ T cell hybridoma, D0-11.10 (●) and its $L3T4^-$ subclone, D0-11.10.AG8.Oua1 (○), were challenged with OVA/I-A^d in the presence of different concentrations of the anti-I-A^d monoclonal antibody, MK-D6. Response was measured as U/ml IL-2 secreted.

by anti-I-A^d antibody, supporting the conclusion that the reaction between the $L3T4^-$ subclone and the Ag/I-A^d-presenting cell occurred with lower overall avidity than if the hybridoma were $L3T4^+$.

Discussion

The problem of how T cells recognize Ag in association with products of the MHC is particularly interesting, not only because of the apparent difficulties in identifying the proteins of the Ag/MHC receptors, but also because of the discovery that other, apparently nonvariable, glycoproteins, contribute to the process. Several theories have been suggested to explain how these nonvariable products contribute; some of these theories have suggested that the nonvariable products bind to monomorphic proteins or monomorphic portions of Class I or Class II molecules, in the case of Leu-2, T8/Lyt-2 or Leu-3, T4/L3T4 proteins, respectively (3, 5–8, 16, 17). The experiments described in this paper set out to test this hypothesis; our results in every case support the idea.

Unfortunately, there is at the moment no easily used method that measures directly the affinity of T cell receptors for Ag and Ag-presenting cells. Although direct binding studies may be used (29–32), we consider these too cumbersome and insensitive to be used in the kind of experiments required for this paper. We therefore measured instead the sensitivity of a series of T cell hybridomas to different Ag doses and to inhibition by anti-I-A^d reagents. Recognition was measured by IL-2 production. IL-2 production in such an assay is undoubtedly dependent on a number of factors, Ag/I receptor density on the T cell hybridoma surface, the strength of the signal that must be delivered to the hybridoma before IL-2 secretion is triggered, and the ease with which the relevant Ag can be generated by the Ag-presenting cell (25); undoubtedly also included amongst these factors is the overall avidity of the T cell for the Ag plus Ag-presenting cell complex, contributed by T cell receptor for Ag/I and by the Ag/I on the presenting cell surface as well as reciprocal interactions between an unknown number of other surface molecules.

Some of the experiments described in this paper showed that a collection of T cell hybridomas specific for the same protein, OVA, in association with the same I-region product, I-A^d, could be ordered according to their sensitivity to OVA concentrations and inhibition by anti-I-A^d. The fact that the rank orders in each type of experiments were similar suggested that an overall avidity of the T cell for Ag/I on the Ag-presenting cell was being measured. We therefore felt justified in using these rank orders to test the hypothesis that the L3T4 contributed to T cell binding to Ag/I on Ag presenting cells by increasing the overall avidity of the cells for each other.

As described before (9), Ag/MHC recognition by T cell hybridomas was variably inhibited by antibodies to L3T4; the degree of inhibition was not related to the amount of L3T4 borne by the T cell. Recognition by some T cell hybridomas was very easily inhibited by the antibodies, but the fact that high concentrations of anti-L3T4 antibodies could hardly affect Ag/MHC recognition by some T cell hybridomas, most notably 3D0-18.3 and 3D0-54.8, suggested that the L3T4 antigen probably did not play an *essential* role in Ag/I recognition by T cells.

The idea that L3T4 simply contributed to the overall avidity of the interaction between T cells and Ag/MHC on Ag-presenting cells was supported by our results, which showed that T cell hybridomas with the lowest sensitivity to Ag/MHC, reflected by their relative unresponsiveness to low Ag doses and by their

greater sensitivity to anti-I antibodies, were the most easily inhibited by anti-L3T4 antibodies. The anti-L3T4 seemed to be interfering with Ag/MHC recognition, not some other event, in the T cell hybridomas because of the ability of the antibody to inhibit binding of a T cell hybridoma to Ag/MHC (9) and because the antibody had no effect on the ability of T cell hybridomas to secrete IL-2 in response to Con A (data not shown).

Subclones of the relatively high affinity T cell hybridoma, D0-11.10, continued to respond to OVA/I-A^d in the absence of an L3T4 molecule. The $L3T4^-$ hybridomas appeared to have the same amount, or more, of receptor for OVA/I-A^d on their surface as their $L3T4^+$ parent, as measured by a monoclonal anti-receptor antibody (19). These results proved that L3T4 is not a determinant on Ag/MHC receptors themselves, a possibility that has been suggested by some investigators (3). In addition, since lack of L3T4 did not interfere with the appearance of Ag/I receptors on our hybridoma, it must not be essential for the surface expression of Ag/I receptors, i.e. it is not an anchoring protein for receptors. The Ag/I receptors on $L3T4^-$ T cell hybridomas appeared to be fully functional, i.e. they could trigger the release of IL-2 by the T cell hybridomas in response to Ag/I, thus the L3T4 molecule does not seem to serve an essential signaling function for the cell.

The properties of the $L3T4^-$ subclones of D0-11.10 did, however, support the hypothesis we were testing in this paper. The subclones did respond less well to OVA/I-A^d than their parent; they apparently had a lower avidity for Ag/I-A^d, since they required more Ag to respond and were inhibited more easily by anti-I-A^d antibodies.

Another point worth noting from the results in this paper is the fact that all the initially cloned T cell hybridomas we tested bore L3T4, Thy-1 and LFA-1. The response of our T cell hybridomas to Ag/MHC does not seem to be affected by anti–Thy-1 monoclonal antibodies and is, to varying degrees, inhibited by anti-LFA antibodies, though only at high concentrations of the monoclonals. Similar results have been noted in the past, using more sensitive normal T cells (33, 34).

The tumor parent for these hybridomas, BW5147, was $L3T4^-$. Although we cannot be sure, the fact that $L3T4^-$ subclones of T cell hybridomas can be isolated with relative ease suggests that L3T4 genes in BW5147 are not induced in T cell hybridomas, rather that only L3T4 genes of the incoming normal T cell parent are expressed. We have isolated subclones of Ag/I $receptor^+$, $L3T4^+$ T cell hybridomas that have lost either the Ag/I receptor (19) or L3T4 (reference 9, and this paper) relatively easily by simply subcloning the hybridoma. For example, apart from the $L3T4^-$ derivatives of D0-11.10 described in this paper, a number of other derivatives of the same hybrid which are also $L3T4^-$ have been made subsequently, with properties similar to those described here for D0-11.10.AG8.Oua1. $L3T4^-$ subclones of the (TG)AL/I-A^d-specific T cell hybridoma, 3DT-18.11, have also been made by our standard procedures. In addition to loss of L3T4, these subclones also lost the ability to respond to (TG)AL/I-A^d, but still responded to Con A. Since we have no monoclonal antibody to the (TG)AL/I-A^d receptor on 3DT-18.11, we do not know whether loss of (TG)AL/I-A^d reactivity of these subclones was due to loss of L3T4, of the

receptor for (TG)AL/I-A^d, or both. Nevertheless the ease with which L3T4$^-$ or Ag/MHC receptor$^-$ subclones can be generated suggested that BW5147 genes do not contribute to either of these entities, as one would expect for the variable portion of the Ag/MHC receptor, of course, and also perhaps that both are allelically excluded on the chromosomes of the normal T cell parent.

Finally, we observed with interest the fact that the D^d-specific T cell hybridoma, 3DT-52.5, bore L3T4. Conventional wisdom would predict that a Class I-specific T cell hybridoma would bear Lyt-2, not L3T4. We do not know whether this unexpected finding reflects a property of the normal T cell parent of the hybridoma, or has something to do with the tumor parent, BW5147. Other hybrid progeny of BW5147 with Class I specificity have been shown to be unexpectedly Lyt-2$^-$ (34), although as far as we know they have not yet been typed for L3T4. Also noteworthy was the fact that the responses of 3DT-52.5 to D^d presented by the I-A^d-bearing B cell lymphoma, A20-1.11, were partially inhibited by anti-L3T4 antibody. We have previously shown that 3DT-52.5 responds better to D^d presented by Ia-bearing lines (26), though 3DT-52.5 has been shown not to be I^d-restricted. It is possible therefore, that the avidity of 3DT-52.5 for D^d-presenting cells is raised by the concomitant interaction of L3T4 for its target molecule.

The target molecule of Leu-3, T4/L3T4 is not known, though all available evidence indicates, as suggested previously, that it is a constant portion of Class II molecules themselves (5). Also we do not understand why T cells should partially, or completely, require the help of this "constant" interaction in order to react properly with Ag/MHC on Ag-presenting cells. Perhaps the low affinity interaction between L3T4 or Lyt-2 and their respective target molecules allow the constant binding to, and screening of, potential Ag/MHC presenting cells by T cells. Nevertheless, the fact that 3DT-52.5 is specific for a Class I molecule, yet bears a T cell molecule usually associated with recognition of Class II products, L3T4, may make this T cell hybridoma particularly useful in sorting out the exact role of L3T4, and identifying the L3T4 target molecule, if any.

Summary

We have examined the role of the murine homologue of Leu-3 T4, L3T4, in recognition of antigen in association with products of the major histocompatibility complex (Ag/MHC) by murine T cell hybridomas. A series of ovalbumin (OVA)/I-A^d-specific T cell hybridomas were ranked in their sensitivity to Ag/I by measuring their ability to respond to low doses of OVA, or their sensitivity to inhibition by anti-I-A^d antibodies. T cell hybridomas with low apparent avidity for OVA/I-A^d, i.e. that did not respond well to low concentrations of OVA and were easily inhibited by anti-I-A^d, were also easily inhibited by anti-L3T4 antibodies. The reverse was true for T cell hybridomas with apparent high avidity for Ag/MHC. We found that the presence of low doses of anti-L3T4 antibodies caused T cell hybridomas to respond less well to low doses of Ag, and to be more easily inhibited by anti-I-A^d antibodies. These results suggested that the role of the L3T4 molecule is to increase the overall avidity of the reaction between T cells and Ag-presenting cells.

In support of this idea was the discovery of several L3T4$^-$ subclones of one of

our L3T4$^+$ T cell hybridomas, D0.11.10. The L3T4$^-$ subclones had the same amount of receptor for OVA/I-A^d as their L3T4$^+$ parent, as detected by an anti-receptor monoclonal antibody. The L3T4$^-$ subclones, however, responded less well to low doses of OVA, and were more easily inhibited by anti-I-A^d antibodies than their L3T4$^+$ parent. These results showed that the L3T4 molecule was not required for surface expression of, or functional activity of, the T cell receptor for Ag/MHC. The L3T4 molecule did, however, increase the sensitivity with which the T cell reacted with Ag/MHC on Ag-presenting cells.

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