

ROLE OF L3T4 IN ANTIGEN-DRIVEN ACTIVATION OF A
CLASS I-SPECIFIC T CELL HYBRIDOMA

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Most T cell receptors for antigen are restricted to recognize antigen in the context of either class I or class II major histocompatibility complex (MHC) antigens. Recently, it has been shown (1–12) that the expression of L3T4/Lyt-2 T cell surface antigens are not simply markers of T cell function, but also appear to be receptors for MHC-encoded antigens. Thus, it appears that L3T4 is a receptor for class II MHC antigens and Lyt-2 is a receptor for class I MHC antigens. Antibodies against these receptors can block function of T cells that express them (1–10), and the function of these receptors appear to be more important in T cell activation when the functional affinity (the use of affinity here is not meant to imply a true affinity, as it is not possible to measure this, but rather the efficacy of antigen stimulation) of the antigen-specific T cell receptor is low (5, 10).

We have previously shown (11, 12) that a class I-specific, L3T4⁺ T cell hybridoma, 3DT52.5, used L3T4 only if the stimulator cell expressed Ia. This and other data (6–10) suggested that L3T4 may directly interact with nonpolymorphic determinants on Ia. Previous data (12) using an L3T4⁻ variant of 3DT52.5 suggested that L3T4 expression was not absolutely required for activation, and provided further evidence for an L3T4/Ia interaction, since the ability to block stimulation with anti-Ia was lost when the hybridoma no longer expressed L3T4. Additionally, we have shown that both functions of the parent hybridoma (to produce interleukin 2 [IL-2] and to act as a cytolytic T cell) demonstrate the same requirements for L3T4, and that there is not a strict requirement for the antigen-specific T cell receptor and the accessory molecule (L3T4 or Lyt-2) on the T cell to be specific for the same class of MHC antigen. It remained unclear why L3T4 was needed for stimulation by an Ia⁺ stimulator, but was not involved in the action of Ia⁻ stimulating cells.

In this report, we have further explored the role of L3T4 and its interaction with Ia molecules. As stimulator cells, we have used L cells transfected with the α and β genes of I-A^k to provide further evidence that Ia-encoded antigens are the ligand for L3T4. With these transfected L cells, we have examined the role of L3T4 in stimulation of a T cell hybridoma under conditions of optimal and

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suboptimal antigen dose. These experiments suggest that when a T cell is suboptimally stimulated, presumably lowering the functional affinity between the antigen-bearing cell and the T cell receptor, an L3T4/Ia interaction is required.

Materials and Methods

Cell Lines. A20.2J.AG (A20) (H-2^d, Ia⁺) was maintained in vitro. Two different cloned lines of transfected fibroblasts were used in these studies. Both are products of transfection of L cells, a C3H fibroblastic tumor cell line made thymidine kinase defective. Transfections were done as previously described (13). The cell line CA25.9.3 was transfected with the H-2D^d-containing cosmid .c18.1 (14). The cell line CA25.8.1 was transfected with the genes respectively coding for the D^d and the I-A^k molecules (13). CA25.8.1 and CA25.9.3 were selected by flow microfluorometry for equal expression of surface H-2D^d. Cells were detached by treatment with versene (Gibco Laboratories, Grand Island, NY)

Monoclonal Antibodies (mAb). mAb were prepared as a cell-free supernatant of hybridoma cultures. mAb used and their specificities are: GK1.5, (L3T4) (6); M5/114, (I-A^{db}, I-E^{dk}) (15); 10.2.16, (I-A^{ks,fr,u}) (16); and 34.5.8 (D^d) (17). The concentration of the antibodies used varied according to experimental protocols, as indicated in Results. Antibodies were used at saturating concentrations.

T Cell Hybridomas. The T hybridomas used in these experiments have been previously described (10–12, 18). Briefly, 3DT52.5 resulted from a fusion of T cell blasts primed with the synthetic polypeptide (T,G)-A-L, and an azaguanine-resistant subline of the AKR thymoma BW5147. 3DT52.5 was characterized as a hybridoma that secretes IL-2 in response to a class I antigen coded for in the H-2D^d region. Two variants of 3DT52.5 were prepared by passaging the cloned hybridoma in tissue culture and recloning the hybrids at limiting dilution. 3DT52.5.8 and 3DT52.5.24 were characterized as IL-2-producing hybridomas with the same antigen specificity as the parent, but which did not express the L3T4 marker.

Culture Conditions. All cell lines were grown and tested in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, penicillin (100 μ g/ml), and streptomycin (100 μ g/ml). For stimulation of IL-2 secretion, 4×10^5 hybridoma cells were cocultured with various numbers of spleen cells or tumor cells (previously exposed to 10,000 rad gamma irradiation) in a total volume of 1 ml. After 20–24 h, supernatants (SN) were removed and assayed for the presence of IL-2.

IL-2 Assay. SN were titrated by serial twofold dilutions and assayed for IL-2 by their ability to support the proliferation of the IL-2-dependent T cell line, HT-2 (19). Proliferation was assessed by the incorporation of [³H]thymidine during a 4-h pulse with 1 μ Ci/well [³H]thymidine. Data are presented as U/ml relative to the [³H]thymidine incorporation of HT-2 from a standard rat concanavalin A (Con A) SN. The half-maximal incorporation from the Con A SN titration was defined as 100 U/ml IL-2. Each table represents a series of at least three experiments.

Results

Previous work (11, 12) has demonstrated that 3DT52.5 is specific for the N/Cl ($\alpha 1/\alpha 2$) domains of H-2D^d. The direct recognition of a class I MHC molecule by an L3T4⁺ T cell hybridoma allowed experimental examination of the role of the L3T4 receptor in the absence of an antigen-specific, class II-restricted T cell receptor. We have shown, in antibody-blocking experiments using L3T4⁻ variants of 3DT52.5, that L3T4 was involved in triggering effector function of 3DT52.5 only in the presence of Ia (12). In this report, we have further investigated whether gene products of the I region encode the ligand for the L3T4 receptor.

A more direct approach to assessing whether Ia genes encode the ligand for L3T4 was the use, as stimulator cells, of L cells transfected with the H-2D^d gene

and the α and β chain genes of I-A^k. CA25.8.1 is a cell line transfected with H-2D^d and α and β genes of I-A^k, and thus expressed H-2D^d and I-A^k, while CA25.9.3 is a cell line transfected with the H-2D^d gene, and thus expressed H-2D^d. These cell lines express very high and equal amounts of H-2D^d, as shown by fluorescence analysis (data not shown). The T cell hybridoma, 3DT52.5, was stimulated with the transfectants. The number of CA25.8.1 and CA25.9.3 stimulator cells was titrated, and the ability to block 3DT52.5 stimulation with anti-L3T4 was examined. This type of experiment (Fig. 1) showed that stimulation of 3DT52.5 with CA25.8.1 (H-2D^d, I-A^k) was sensitive to blocking by anti-L3T4 with 10^4 or fewer stimulator cells, but not at 3×10^4 antigen-bearing cells. Stimulation of 3DT52.5.8 or 3DT52.5.24 is not sensitive to anti-L3T4 blocking at any dose of CA25.8.1 (data not shown). CA.25.9.3 stimulation (H-2D^d) of 3DT52.5 was not affected by anti-L3T4 at any tested dose of antigen. These experiments suggest that the requirement for L3T4 in activation is based on the relative ability of a cell to stimulate a T cell hybridoma. If a stimulator cell bears enough antigen for optimal stimulation, L3T4 may not be required.

Another approach to this question was undertaken by examining the effect of lowering the H-2D^d density on the stimulating cells. 3DT52.5 was stimulated with either the H-2D^d B cell lymphoma, A20 (H-2D^d, Ia⁺), or L cells transfected with H-2D^d or H-2D^d and I-A^k. This experiment (Table I) shows that 3DT52.5 stimulation by a high number of transfected cells, CA.25.8.1 and CA25.9.3, was not affected by anti-L3T4 or anti-Ia, while A20 stimulation was blocked by both anti-L3T4 and anti-Ia. This may be reflected in the amount of H-2D^d on the various cells used. A20 expressed five-to seven-fold less H-2D^d, as detected in a radioimmunoassay, when compared to the transfected L cells (data not shown). We hypothesized that, at high concentrations of H-2D^d, the functional affinity of 3DT52.5 for antigen was high enough to result in T cell triggering in the absence of an L3T4/Ia interaction.

To further test this hypothesis, the amount of H-2D^d on CA25.8.1 and CA25.9.3 was functionally lowered by the addition of 1/5 the saturating concentration of anti-H-2D^d mAb. Decreasing the H-2D^d antigen density by the addition of mAb revealed the ability of anti-L3T4 (Table I) and anti-Ia (not shown) to block stimulation by CA25.8.1 (the H-2D^d- and I-A^k-transfected L cell) but not CA25.9.3 (the H-2D^d-transfected L cell). Although anti-L3T4 only partially blocked in the presence of a suboptimal amount of anti-H-2D^d, this was seen in three separate experiments, and there was at least a 50% decrease in the amount of IL-2 produced. 3DT52.524 (an L3T4⁻ variant of 3DT52.5) was not blocked by either anti-L3T4 or anti-Ia under any of the conditions tested. This experiment implied that if 3DT52.5 is stimulated by a suboptimal dose of H-2D^d, the L3T4/Ia interaction becomes involved in the subsequent triggering of IL-2 production. Additionally, the experiments shown in Fig. 1 and Table I suggest that the I-A^k plus H-2D^d-transfected cell (CA 25.8.1) stimulates more IL-2 at lower doses of antigen than the H-2D^d-transfected L cell.

Discussion

We have addressed the question of the specificity of L3T4 by studying a class I-specific, L3T4⁺ T cell hybridoma, 3DT52.5. This hybridoma has allowed the separate evaluation of the T cell receptor and L3T4. The Ia specificity of L3T4

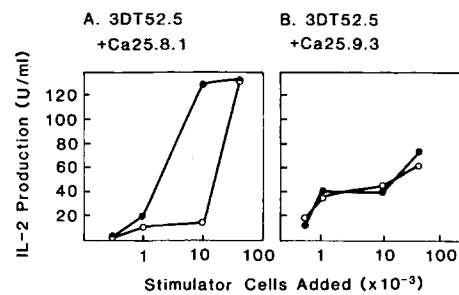


FIGURE 1. Anti-L3T4 blocking of IL-2 production is dependent on the relative number of Ia⁺ stimulator cells. IL-2 production by 3DT52.5 after stimulation by varying numbers of CA25.8.1 (A) or CA25.9.3 (B), in the presence (open symbols) or absence (closed symbols) of anti-L3T4.

TABLE I
L3T4/Ia Interaction Is Required When Antigen Is Limiting

mAb against*	IL-2 production with 3DT52.5 plus:			IL-2 production with 3DT52.5.24 plus:	
	A20 (D ^d Ia ^d)	CA25.8.1 (D ^d Ia ^k)	CA25.9.3 (D ^d)	CA25.8.1 (D ^d Ia ^k)	CA25.9.3 (D ^d)
---	100 [‡]	100	100	100	100
L3T4	29	102	83	115	91
D ^d	20	19	5	55	75
Ia ^k	ND [§]	119	83	102	85
D ^d I	ND	71	25	81	85
D ^d plus L3T4 [†]	ND	42	25	80	84

* mAb used at 10%.

[‡] The amount of IL-2 was determined in an HT2 assay, as described, and the amount produced in the absence of mAb was normalized to 100%. 4×10^5 hybridoma cells were stimulated with 6×10^5 A20, CA25.8.1, or CA25.9.3 for 24 h, as described. Amount of IL-2 produced was as follows: 3DT52.5 plus A20, 334 U/ml; plus CA25.8.1, 144 U/ml; plus CA25.9.3, 240 U/ml. With 3DT52.5.24 plus CA25.8.1, 1,000 U/ml; plus CA25.9.3, 1175 U/ml.

[§] Not done.

[†] Using $\frac{1}{3}$ the saturating concentration of anti-H-2D^d.

can be implied from our previous experiments (12), and the recent observation that planar membranes containing only purified I-A^d and ovalbumin fragments stimulated an I-A^d-restricted, ovalbumin-specific T cell hybridoma, and could be blocked by anti-L3T4 (20).

Another approach to the evaluation of the L3T4 ligand is the use of L cells transfected with I-A genes. We were interested in directly assessing the role of L3T4 in activation of 3DT52.5 by stimulator cells showing equal expression of H-2D^d. CA25.9.3 and CA25.8.1 stimulated IL-2 production by 3DT52.5; however, anti-L3T4 and anti-Ia blocked stimulation only by the L cell transfected with I-A^k. At high doses of antigen, it appears that the L3T4/Ia interaction is not required to stimulate 3DT52.5. Therefore, by both partial antibody blocking of the available surface H-2D^d, and by titration of the stimulator cells, we have shown that when 3DT52.5 is stimulated by a suboptimal dose of antigen, the L3T4/Ia interaction becomes involved in activation. In fact, it appears that 3DT52.5 makes more IL-2 when stimulated with suboptimal amounts of antigen in the presence of I-A^k than in the absence of I-A, implying an increase in activation per cell. These experiments suggest that the L3T4/Ia interaction

becomes important in T cell stimulation when the T cell's interaction with antigen is limited by either the amount of antigen present or the functional affinity of the T cell antigen-specific receptor. The ability of transfected I-A to interact with L3T4 adds further evidence for a direct interaction between Ia and L3T4, although it remains possible that an Ia-associated molecule is the ligand.

The involvement of L3T4 in most class II-restricted T cell activation has implicated L3T4 in the recognition of Ia. This interaction appears to be required during antigen recognition (6-8), but not for effector function. The data presented here demonstrate the ability of L3T4 to interact with transfected I-A, and clarify the role of the L3T4/Ia interaction in T cell activation. It appears that L3T4/Ia interaction is not essential for all T cell activation, and may become necessary for T cell triggering with low functional-affinity T cell interactions.

Summary

The expression of L3T4/Lyt-2 on murine T cells has led to the association of these surface markers with recognition of either class II or class I major histocompatibility complex (MHC) antigens. It has been suggested that these T cell surface antigens interact with nonpolymorphic determinants on MHC antigens. We have examined the role of L3T4 in the recognition of H-2D^d by the T cell hybridoma, 3DT52.5. Mouse L cells transfected with either the H-2D^d gene, or with both the α and β genes of I-A^k and the H-2D^d gene have been used to assess the role of an L3T4/Ia interaction at varying doses of H-2D^d. A role of L3T4 in activation of 3DT52.5 becomes evident only at limiting doses of antigen. It appears that an L3T4/Ia interaction can influence T cell function during suboptimal stimulation, implying that the L3T4/Ia interaction serves to raise the functional affinity of interaction between the T cell and the antigen-bearing cell.

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References

1. Nakayama, E., H. Shiku, E. Stockett, H. F. Oettgen, and L. J. Old. 1979. Cytotoxic T cells: Lyt phenotype and blocking of killing activity by Lyt antisera. *Proc. Natl. Acad. Sci. USA.* 76:1977.
2. Hollander, N., E. Pillemer, and I. L. Weissman. 1980. Blocking effect of Lyt-2 antibodies on T cell functions. *J. Exp. Med.* 152:674.
3. Shinohara, N., U. Hammerling, and D. Sachs. 1980. Mouse alloantibodies capable of blocking cytotoxic T cell function. II. Further study on the relationship between the blocking antibodies and the products of the Lyt-2 locus. *Eur. J. Immunol.* 10:589.
4. Swain, S. L. 1981. Significance of Lyt phenotypes: Lyt-2 antibodies block activities of T cells that recognize class I major histocompatibility complex antigens regardless of their function. *Proc. Natl. Acad. Sci. USA.* 78:7101.
5. MacDonald, H. R., A. L. Glasebrook, C. Bron, A. Kelso, and J. C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt-2,3 molecules on cytotoxic

- T lymphocytes (CTL): possible implications for the affinity of CTL antigen receptors. *Immunol. Rev.* 68:69.
6. Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Havran, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch. 1983. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1.5; expression of L3T4a by functional T cell clones appears to correlate primarily with Class II MHC antigen-reactivity. *Immunol. Rev.* 74:29.
 7. Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Pierres, J. Quintans, M. R. Loken, M. Pierres, and F. W. Fitch. 1984. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu3/T4 molecule. *J. Immunol.* 131:2445.
 8. Wilde, D. B., P. Marrack, J. Kappler, D. P. Dialynas, and F. W. Fitch. 1983. Evidence implicating L3T4 in class II MHC antigen reactivity: monoclonal antibody GK1.5 (anti-L3T4a) blocks class II MHC antigen-specific proliferation, release of lymphokines, and binding by cloned murine helper T lymphocyte lines. *J. Immunol.* 131:2178.
 9. Swain, S. L., D. Dialynas, F. W. Fitch, and M. English. 1984. Monoclonal antibody to L3T4 blocks the function of T cells specific for class 2 major histocompatibility complex antigens. *J. Immunol.* 132:118.
 10. Marrack, P., R. Enders, R. Shimonkevitz, A. Zlotnik, D. Dialynas, F. Fitch, and J. Kappler. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product. *J. Exp. Med.* 158:1077.
 11. Endres, R., P. Marrack, and J. W. Kappler. 1983. An IL-2-secreting T cell hybridoma that responds to a self class I histocompatibility antigen in the H-2D region. *J. Immunol.* 131:1656.
 12. Greenstein, J. L., J. Kappler, P. Marrack, and S. J. Burakoff. 1984. The role of L3T4 in recognition of Ia by a cytotoxic, H-2D^d-specific T cell hybridoma. *J. Exp. Med.* 159:1213.
 13. Malissen, B., M. Peele-Price, J. M. Goverman, M. McMillan, J. White, J. Kappler, P. Marrack, A. Pierres, M. Pierres, and L. Hood. 1984. Gene transfer of H-2 class II genes: antigen presentation by mouse fibroblast and hamster B-cell lines. *Cell.* 36:319.
 14. Steinmetz, M., A. Winoto, K. Minard, and L. Hood. 1982. Clusters of genes encoding mouse transplantation antigens. *Cell.* 28:489.
 15. Bhattacharya, A., M. E. Dorf, and T. A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* 127:2488.
 16. Oi, V., P. Jones, J. Goding, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. *Contemp. Top. Microbiol. Immunol.* 81:115.
 17. Ozato, K., N. M. Mayer, and D. H. Sachs. 1982. Monoclonal antibodies to mouse MHC antigens. IV. A series of hybridoma clones producing anti-H-2^d antibodies and an examination of expression of H-2^d antigens on the surface of these cells. *Transplantation (Baltimore)*. 34:114.
 18. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198.
 19. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* 150:1510.
 20. Watts, T. H., A. B. Brian, J. W. Kappler, P. Marrack, and H. M. McConnell. 1984. Antigen presentation by supported planar membranes containing affinity purified I-A^d. *Proc. Natl. Acad. Sci. USA.* 81:7564.