

## ROLE OF Ly-6 IN LYMPHOCYTE ACTIVATION

### II. Induction of T Cell Activation by Monoclonal Anti-Ly-6 Antibodies

BY THOMAS R. MALEK, GUSTAVO ORTEGA, CHRISTINA CHAN,  
RICHARD A. KROCZEK, AND ETHAN M. SHEVACH

*From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases,  
National Institutes of Health, Bethesda, Maryland 20892*

The physiological activation of T lymphocytes requires the triggering of the disulfide-linked, 90-kD  $\alpha/\beta$  heterodimer of the T cell receptor after its recognition of antigen that is displayed on the cell surface of a suitable APC in the context of either an MHC class I or class II gene product (1, 2). The T cell receptor (Ti)<sup>1</sup> is part of a molecular complex that contains four distinct, noncovalently associated polypeptide chains, some of which are reactive with anti-T3 mAbs in man (3, 4). An obligatory requirement for coexpression of the Ti and T3 exists for activation of T lymphocytes (5, 6). In addition to the T3-Ti complex, the T11 molecule (7) and the 44-kD polypeptide identified by human mAb 9.3 (8, 9), as well as Thy-1 (10–12) and the recently described murine 12-kD TAP protein (13), have each been implicated in playing a role in the T cell activation process, since mAbs against all of these structures can be mitogenic. The putative function of this latter group of cell surface molecules is poorly understood, although they may function either to amplify or modify signals generated via the T3-Ti complex, or may serve as distinct alternative pathways for T cell activation, as has been suggested for T11 (7, 14).

The development and use of mAbs have permitted the definition of a number of other cell surface molecules that are expressed on T cells, but in many cases the potential role of these molecules in T cell function is not known because these mAbs do not affect T cell functions when added continuously to culture, and they do not appear to be markers for functional subpopulations of T cells. In the mouse, one such group of cell surface molecules whose function is not understood is the Ly-6 alloantigens. The Ly-6 locus is found on murine chromosome 2, and it controls the expression and/or encodes for alloantigenic specificities found primarily on T and B lymphocytes (15). Two haplotypes, Ly-6.1 and Ly-6.2, map to the Ly-6 locus. As many as five distinct Ly-6 alloantigens

---

Dr. Kroczek was supported in part by a grant from the Deutsche Forschungsgemeinschaft. Dr. Ortega was supported by a fellowship from CONICET. Dr. Kroczek's present address is Abteilung Fur Abdominal und Transplantations Chirurgie, University of Hannover, Hannover, West Germany. Dr. Ortega's present address is Instituto De Biomedicina, Caracas, Venezuela. Dr. Malek's address is Department of Microbiology, University of Miami, School of Medicine, Miami, FL 33101. Address correspondence to E. M. Shevach, Laboratory of Immunology, National Institutes of Health, Building 10, Room 11N311, Bethesda, MD 20205.

<sup>1</sup> *Abbreviations used in this paper:* AC, accessory cells; BINS, beef insulin; [Ca<sup>2+</sup>]<sub>i</sub>, cytoplasmic free calcium; PINS, pork insulin; RARig, rabbit anti-rat Ig; TAP, T cell-activating protein; Ti, T cell receptor.

may exist for each haplotype (16–18), and mAbs are available that are specific for Ly-6.2A, B, C, and D, and Ly-6.1E. However, the distinction of Ly-6A–E specificities largely resides in different cell distributions of the determinants detected by these mAbs. Detailed biochemical and molecular analyses of Ly-6 molecules and genes are necessary to establish precisely the nature of the specificities detected by these antibodies, and whether Ly-6 may represent a multigene complex rather than a single gene (19).

We have recently identified and characterized a new rat mAb, D7, that recognizes a nonpolymorphic Ly-6 specificity.<sup>2</sup> Serological and biochemical studies indicated that D7 defined a determinant on molecules bearing Ly-6E.1 specificities in Ly-6.1<sup>+</sup> strains, as well as a determinant on molecules bearing Ly-6A.2 in Ly-6.2<sup>+</sup> strains. In the present report, we will show that after crosslinking, mAb D7 is a potent inducer of T cell activation, as assayed by augmentation of antigen- and alloantigen-induced T cell proliferation, by induction of IL-2 production by T cell hybridomas, by induction of resting T cell proliferation in the presence of the costimulator, PMA, and by induction of a rise in cytoplasmic-free calcium [Ca<sup>2+</sup>]<sub>i</sub>. Furthermore, a number of other well-characterized mAbs to Ly-6 locus products also appear to be capable of inducing T cell activation. Collectively, these findings raise the possibility that an Ly-6 molecule(s) may play a critical role in the T cell activation cascade.

### Materials and Methods

*Animals.* Mice were obtained from the Animal Production Service, National Institutes of Health.

*Antibodies.* Production and characterization of mAb D7 to a nonpolymorphic Ly-6 specificity has been described in detail elsewhere.<sup>2</sup> The anti-Ly-6 mAbs 34-11-3 (17), S8.106 (18), and 6C3 (20) have also been previously characterized. mAb 109-9-17, which is directed to Ly-6C.2, was the generous gift of Dr. J. Bluestone, NIH. The properties of mAbs H10-13-4 to Thy-1.2 (21), Gk1.5 to L3T4 (22), 19/178C to Ly-2.2 (23), Fd441.8 to LFA-1 (24), M5/114 (25) to Ia, 7D4 and 3C7 to the IL-2-R (26, 27), M1/42.3.9.8 to H-2 (28), M1/9.3.4.HL.2 to the common leukocyte antigen (29), 24G2 to the Fc receptor (30), and MAR 18.5 to rat  $\kappa$  light chains (31) have all been described previously. Rabbit anti-rat Ig serum (RARIG) was produced by immunizing a rabbit with rat  $\gamma$ -globulin fraction (Cappel Laboratories, Cochranville, PA). 1 mg of protein was injected subcutaneously in CFA, followed by a boost 3 wk later with 0.4 mg injected intramuscularly in IFA; after two additional boosts every 60 d the rabbit was bled repeatedly. The RARIG serum was highly crossreactive with mouse Ig. Goat anti-rat IgG sera were purchased from Cappel Laboratories.

*Purification of D7.* D7 was purified on an immunoaffinity column containing MAR 18.5. After elution with 3.5M MgCl<sub>2</sub>, the antibodies were extensively dialyzed in 0.01 M PBS, pH 7.4, and was then filter-sterilized.

*Culture Medium.* All cultures were performed in RPMI 1640 (Biofluids Inc., Rockville, MD) supplemented with glutamine (300  $\mu$ g/ml), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 2-ME (5  $\times$  10<sup>-5</sup> M), and 10% FCS.

*T Cell Hybridomas.* Antigen-specific T cell hybridomas were produced by fusion of immune T cells to BW5147, as previously described (32). To induce IL-2 secretion, the T cell hybridomas (10<sup>5</sup>) were cultured in 0.2 ml of medium in 96-well microtiter plates (3596; Costar, Cambridge, MA), with or without the various stimuli, for 24–48 h. The

<sup>2</sup> Ortega, G., P. E. Kory, E. M. Shevach, and T. R. Malek. Role of Ly-6 in lymphocyte activation. I. Characterization of a monoclonal antibody to a non-polymorphic Ly-6 specificity. Submitted for publication.

IL-2 activity in the culture supernatants was detected by its capacity to stimulate the proliferation of an IL-2-dependent line, CTL-L.

*Cell Purification and Culture Conditions.* T lymphocytes were isolated from spleens, peripheral lymph nodes, or mesenteric lymph nodes (33) by collection of nylon wool nonadherent cells. Nonimmune T cells were used in all experiments, except in studies that measured antigen-specific proliferation, in which case the mice were previously immunized in the foot pads with 50  $\mu$ g beef insulin (BINS) emulsified in CFA. AC depletion of the T cell populations was accomplished by treatment with anti-Ia antibody (M5/114) and C (33). Isolation of L3T4<sup>+</sup> or Ly-2<sup>+</sup> T cells was accomplished by treatment of the T cells with either anti-Ly-2.2 (19/178C) (23) or anti-L3T4 (Gk1.5), respectively, and C as previously described (33). AC were either anti-Thy-1.2 + C-treated, irradiated (2,500 rad) spleen cells or irradiated (10,000 rad) LB cells, a B cell hybridoma with antigen presenting capacity (34). The cells were cultured with the various stimuli in 0.2 ml of medium in 96-well microtiter plates. The exact culture conditions for individual experiments are described in the legends to the Figures and Tables. Proliferation of the cultures was measured by adding [<sup>3</sup>H]TdR (New England Nuclear, Boston, MA) during the last 16 h of culture, and then harvesting the cells with a semiautomated device (Ph.d, Cambridge, MA). Data are expressed as the mean of triplicate cultures. The SEM was generally <10% of the mean.

*Determination of [Ca<sup>2+</sup>]<sub>i</sub>.* In a modification of the method described by Tsien et al. (35–37), the cells to be assayed ( $5 \times 10^6$ /ml) were loaded in complete culture medium with a final concentration of 15  $\mu$ M Quin 2-AM (Sigma Chemical Co., St. Louis, MO) for 20 minutes. After loading, the cells were washed two times with Dulbecco's PBS (Biofluids, Inc.), resuspended in the same buffer to a concentration of  $5 \times 10^6$  cells/ml, and were used immediately after equilibration at 37°C for 5 min. When indicated, Quin-2-loaded T cells ( $10^7$ ) were incubated with mAb D7 (250  $\mu$ l of 1:10 ascites) for 15 min at 37°C, and were washed again two times. Fluorescence intensity was measured with a Perkin Elmer fluorescence spectrophotometer LS-5 (excitation, 339 nm; emission, 492 nm). The cuvette chamber was maintained at 37°C, and the cell suspension was continuously stirred. After a baseline was established, goat anti-rat IgG serum (1/100) was added. Maximum fluorescence ( $F_{\max}$ ) was determined by lysing the Quin-2-loaded cells with 0.1% Triton X-100. Minimum fluorescence ( $F_{\min}$ ) was obtained after addition of EGTA (4 mM) and sufficient Tris-buffer to raise the pH to 8.0 (to optimize chelation of Ca<sup>2+</sup> by EGTA). Approximate values for cytoplasmic-free Ca<sup>2+</sup> were calculated by the formula:  $[Ca^{2+}]_i = 115 \text{ nM } (F_x - F_{\min}) / (F_{\max} - F_x)$ .

## Results

*D7 Activates Normal T Cells in the Presence of PMA and AC.* The D7 mAb defined a nonpolymorphic specificity that mapped to the Ly-6 locus and reacted with an epitope on 20–30% of spleen cells from Ly-6.1 mice (e.g., BALB/c), and on 50–70% of spleen cells from Ly-6.2 mice (e.g., C57BL/6). Mitogen stimulation of the spleen cells resulted in the expression of high levels of D7 bearing Ly-6 molecules on all activated T and B lymphocytes, regardless of the strain.<sup>2</sup> When nylon column-passed, anti-Ia- and C-treated, lymph node T cells were cultured with D7 in the presence or absence of PMA (Table I), we did not see a significant proliferative response. However, when the cultures were supplemented with T cell-depleted spleen cells as a source of AC, we saw a vigorous proliferative response, but only when PMA was present. Thus, the stimulation of resting T cell proliferation by mAb D7 required two costimulatory signals PMA and AC function provided by T cell-depleted spleen cells. The stimulatory capacity of mAb D7 was not restricted to one of the major T cell subsets as both L3T4<sup>+</sup> and Ly-2<sup>+</sup> T cells showed equivalent proliferative responses when challenged with D7 and PMA in the presence of AC (Fig. 1).

TABLE I  
*D7 Activates Peripheral T Lymphocytes in the Presence of  
 PMA and AC*

| Stimulus        | AC | [ <sup>3</sup> H]TdR incorporation |         |
|-----------------|----|------------------------------------|---------|
|                 |    | Exp. 1                             | Exp. 2  |
| Medium          | -  | 999                                | 845     |
|                 | +  | 1,324                              | 1,222   |
| PMA (10 ng/ml)  | -  | 2,545                              | 5,949   |
|                 | +  | 3,579                              | 6,577   |
| D7 (10 μg/ml)   | -  | 487                                | 1,112   |
|                 | +  | 823                                | 1,496   |
| D7 + PMA        | -  | 6,747                              | 15,706  |
|                 | +  | 129,325                            | 197,033 |
| Con A (5 μg/ml) | -  | 3,259                              | 426     |
|                 | +  | 33,940                             | 78,114  |

Nylon column-passed mesenteric lymph node cells were treated with anti-Ia and C and then cultured ( $10^5$  cells/well) in the presence or absence of T cell-depleted spleen cells ( $10^5$  cells/well) as a source of AC. [<sup>3</sup>H]TdR incorporation was determined after 72 h of culture; each value is the mean of three determinations.

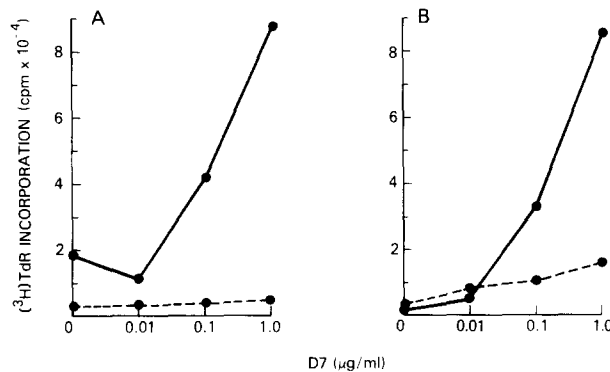


FIGURE 1. Proliferation of L3T4<sup>+</sup> and Ly-2<sup>+</sup> T cells to D7 is AC-dependent. Highly purified AC-depleted L3T4<sup>+</sup> (A) or Ly-2<sup>+</sup> (B) T cells ( $2 \times 10^5$ ) from BALB/c mice were cultured with PMA (10 ng/ml), and the indicated concentration of purified D7 in the presence (—) or absence (---) of normal AC ( $2 \times 10^5$ ) for 72 h.

The stimulation of purified T cells by the combination of D7, PMA, and AC resulted in both IL-2 production in the culture supernatants 24 h after culture initiation, as well as IL-2-R expression, as determined by staining with an mAb to the IL-2-R (7D4) and analysis by flow cytometry (results not shown). Neither IL-2 production nor IL-2-R expression was observed unless all three stimulatory signals were present. The proliferative response seen after 72 h of culture was readily inhibited by addition of the anti-IL-2-R mAb to the culture (Fig. 2). Taken together, these results indicate that D7-induced T cell proliferation occurs by an IL-2-dependent pathway.

*D7 Induces IL-2 Secretion by T Cell Hybridomas.* We have shown elsewhere that BW5147, as well as several T cell hybridomas that were generated by fusion of activated T cells to BW5147, expressed Ly-6 molecules as defined by D7.<sup>2</sup>

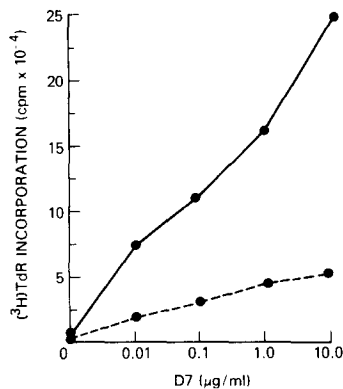


FIGURE 2. Effect of anti-IL-2-R antibodies on D7-induced proliferation. BALB/c T cells ( $2 \times 10^5$ ) were cultured with PMA (10 ng/ml) and the indicated concentration of D7 in the presence of 1:500 dilution of control ascites (—, 50C1 and 49C2) or anti-IL-2-R antibodies (---, 3C7 and 7D4) for 72 h.

TABLE II  
*D7 Stimulates IL-2 Production by T Cell Hybridomas in the Absence of PMA but in the Presence of AC*

| Hybridoma | AC        | $[^3\text{H}]\text{TdR}$ of CTL-L (cpm) |                                 |                                  |        |
|-----------|-----------|---|---------------------------------|----------------------------------|--------|
|           |           | Medium                                  | D7 (1 $\mu\text{g}/\text{ml}$ ) | D7 (10 $\mu\text{g}/\text{ml}$ ) | Con A  |
| 6-3       | None      | 3,614                                   | 2,431                           | 3,584                            | 12,096 |
|           | Spleen AC | 1,610                                   | 1,388                           | 1,613                            | 13,760 |
|           | LB cells  | 1,540                                   | 14,571                          | 26,514                           | 44,304 |
| 4-7       | None      | 2,200                                   | 1,759                           | 1,350                            | 38,037 |
|           | Spleen AC | 2,992                                   | 1,895                           | 2,055                            | 53,517 |
|           | LB cells  | 1,040                                   | 47,861                          | 59,380                           | 62,626 |
| 4-1       | None      | 6,676                                   | 8,462                           | 4,651                            | 33,426 |
|           | Spleen AC | 4,555                                   | 867                             | 3,522                            | 30,750 |
|           | LB cells  | 1,212                                   | 53,776                          | 54,487                           | 61,990 |
| A22       | None      | 837                                     | 1,296                           | 1,150                            | 23,023 |
|           | Spleen AC | 848                                     | 771                             | 887                              | 24,777 |
|           | LB cells  | 3,115                                   | 13,048                          | 25,547                           | 51,773 |

T cell hybridomas ( $10^5$  cells/well) were cultured with D7 or Con A (5  $\mu\text{g}/\text{ml}$ ) in the presence of irradiated (10,000 rad) LB cells ( $2.5 \times 10^4$ /well) or irradiated (2,500 rads) T cell-depleted spleen cells ( $10^5$  cells/well). After a 48 h culture, the IL-2 content of the supernatants was assayed by culture on the CTL-L cell line.

Therefore, we wished to test whether mAb D7 would induce IL-2 secretion by these hybridoma lines (Table II). Since D7 had no effect on the proliferative response of CTL-L cells in the presence or absence of added IL-2, it was possible to measure IL-2 levels in supernatants that contained D7. D7 induced the production of significant amounts of IL-2 from all T cell hybridomas tested in the absence of PMA; no IL-2 secretion occurred when BW5147 was cultured with D7 (not shown). However, in contrast to the response to the mitogen Con A, D7-induced IL-2 secretion required that the cultures be supplemented with LB cells (34). Under these conditions, the amount of IL-2 that was secreted was comparable to that induced by Con A. Presumably, the role of LB cells in the

TABLE III  
*AC Function for D7 Stimulation Is Fc Receptor-mediated*

| Exp. | Responding cell      | Stimulus      | cpm          |           |        |
|------|----------------------|---------------|--------------|-----------|--------|
|      |                      |               | Without 24G2 | With 24G2 |        |
| 1    | T cell hybrids*      | AF3G7         | LB + BINS    | 20,194    | 20,266 |
|      |                      |               | LB + D7      | 44,262    | <100   |
|      | P4.4                 | LB + PINS     | 89,133       | 133,778   |        |
|      |                      | LB + D7       | 21,276       | 1,867     |        |
|      | A22                  | LB + OVA      | 13,510       | 20,817    |        |
|      |                      | LB + D7       | 37,687       | 7,813     |        |
| 2    | T cells <sup>†</sup> | PMA           | 35,689       | 27,256    |        |
|      |                      | PMA + D7 + AC | 165,806      | 30,682    |        |

\* The indicated T cell hybridoma ( $10^5$ ) was cultured with LB ( $2.5 \times 10^4$  cells/well) and D7 ( $10 \mu\text{g/ml}$ ) or BINS ( $10 \mu\text{g/ml}$ ), pork insulin (PINS,  $10 \mu\text{g/ml}$ ), or OVA ( $50 \mu\text{g/ml}$ ) in the presence or absence of the anti-Fc receptor antibody (24G2, 5% culture supernatant) for 48 h. The cpm represent IL-2 production as assayed on CTL-L cells.

<sup>†</sup> Nylon column-passed BALB/c T cells ( $2 \times 10^5$ ) were cultured with PMA ( $10 \text{ ng/ml}$ ) and D7 ( $10 \mu\text{g/ml}$ ) in the presence or absence of an anti-Fc receptor antibody (24G2) for 72 h. Results are expressed as cpm [ $^3\text{H}$ ]TdR incorporation.

cultures was to provide an AC function. In view of the ability of anti-Thy-1 and C-treated spleen cells to function as AC for D7-induced proliferation of normal spleen T cells, it was surprising that such normal AC populations failed to function with D7 to induce IL-2 secretion by the T cell hybrids. Thus, these results indicated that the induction of IL-2 secretion by D7 was AC-dependent, and they suggested that a qualitative or quantitative difference existed in the capacity of different cell populations to serve as AC.

*Fc-mediated Crosslinking of D7 Is Required for T Cell Activation.* One possible role for the AC in D7-mediated T cell activation is that the Fc receptor on the AC is required for crosslinking of D7 bound to the responder T cell. To directly test this possibility, we examined whether an mAb (24G2) to the murine Fc receptor (30) could inhibit D7-induced IL-2 secretion by antigen-specific T cell hybridomas in the presence of LB cells (Table III, Exp. 1). In each instance, IL-2 secretion by several T cell hybridomas in response to D7 was markedly inhibited by 24G2. As an internal specificity control, 24G2 had no inhibitory effects on antigen-induced IL-2 secretion by these same hybridomas in the presence of LB cells. 24G2 also inhibited the proliferation of splenic T cells to D7 and PMA (Table III, Exp. 2). However, 24G2 did not inhibit the background proliferative response of the T cells to PMA alone, which in this particular experiment was somewhat higher than usually seen. This result indicated that Fc receptor-mediated function was also necessary for D7-induced mitogenesis of normal T cells.

We next evaluated whether crosslinking of D7 bound to the responder cell surface with anti-Ig could also induce T cell activation in the presence of PMA, but in the absence of AC. Highly purified, AC-depleted T cells were pretreated with D7 or a variety of other mAbs with different specificities. The T cells were then cultured with PMA in the presence or absence of a rabbit anti-rat Ig serum

TABLE IV  
*The AC Cell Requirement for D7 Stimulation Can Be Bypassed by the Use of a Crosslinking Second Antibody*

| Pretreatment              | $\Delta$ cpm [ $^3\text{H}$ ]TdR incorporation |                   |
|---------------------------|--|-------------------|
|                           | PMA  | PMA + Anti-rat Ig |
| Medium                    | 2,414  | 4,261             |
| D7                        | 5,024  | 341,871           |
| LFA-1                     | 3,704  | 2,321             |
| H-2                       | 4,014  | 14,976            |
| CLA                       | 3,453  | 5,217             |
| Ia                        | 2,821  | 2,045             |
| L3T4                      | 2,891  | 4,588             |
| Ly-2                      | 2,149  | 4,049             |
| Continuous D7 + PMA,      | 5,928  |                   |
| Continuous D7 + PMA + AC, | 256,550  |                   |

Highly purified AC-depleted T cells ( $2 \times 10^5$ ) from BALB/c mice were pretreated by incubation with an excess of the indicated antibody, were washed, and then cultured with PMA (10 ng/ml) and 1:500 dilution of rabbit anti-rat Ig antiserum for 72 h as indicated. Results are expressed as  $\Delta$  cpm [ $^3\text{H}$ ]TdR incorporation [(cpm [ $^3\text{H}$ ]TdR incorporation in the presence of stimulus) - (cpm in medium alone)]. Background responses in the presence of medium alone were  $<2,000$  cpm.

(Table IV). A significant proliferative response was seen after antibody-induced crosslinking of D7 in the presence of PMA. This proliferative response was comparable to the response from cultures that contained AC, continuous D7, and PMA. In contrast, no proliferation was seen after crosslinking other antibodies reactive with the responding T cells, with the possible exception of a small degree of proliferation when the cells were pretreated with mAb M1/42.3.9.8 directed to a framework determinant of H-2.

*Effect of D7 on Alloantigen-, Antigen-, and Mitogen-induced T Cell Proliferation.* The addition of purified D7 caused a dose-dependent enhancement of the MLR (Fig. 3A) and antigen-specific T cell proliferative responses to BINS and PPD (Fig. 3B). Although minimal proliferation was noted when T cells were cultured with syngeneic stimulator cells, the addition of D7 also permitted the detection of a syngeneic MLR (SMLR) (Fig. 3A). D7 enhanced proliferation in these assays when T cells from BALB/c and C57BL/6 mice were analyzed, indicating that T cells from both Ly-6.1 and Ly-6.2 mice were affected by D7. Dose-response studies showed that as little as 10 ng/ml of D7 was needed to cause detectable enhancement of proliferation, although maximal responses were detected at 10  $\mu\text{g}/\text{ml}$  of antibody. Surprisingly, D7 failed to enhance proliferation when T cells were stimulated with the mitogens Con A and PHA (Fig. 3C). This failure of D7 to augment mitogen-induced proliferation was also seen when a suboptimal concentration of Con A (Fig. 3C) or PHA (not shown) was used to stimulate the T cells. We never observed inhibitory effects of D7 on T cell activation under a variety of culture conditions.

*Other Anti-Ly-6 mAbs Induce T Cell Activation.* Although the studies performed thus far have all used the anti-Ly-6 mAb, D7, we wished to evaluate other well-

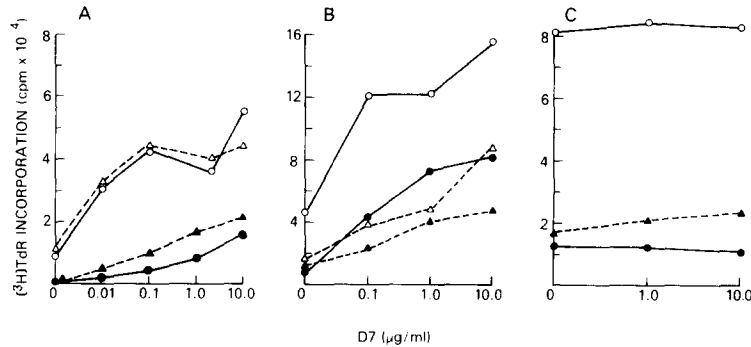


FIGURE 3. Effect of D7 on T lymphocyte proliferation. (A) Effect on the MLR and syngeneic MLR. Nylon wool-purified T lymphocytes from mesenteric lymph node ( $2 \times 10^5$ ) were cultured with syngeneic or allogeneic anti-Thy-1.2 + C-treated irradiated (2,500 rad) spleen cells ( $2 \times 10^5$ ) for 5 d. The cultures were BALB/c anti-BALB/c (●), BALB/c anti-C57BL/6 (○), C57BL/6 anti-C57BL/6 (▲), and C57BL/6 anti-BALB/c (△). (B) Effect on antigen-induced proliferation. Nylon wool-purified lymph node T cells ( $2 \times 10^5$ ) from BALB/c mice immunized with BINS were cultured with BINS (10  $\mu\text{g}/\text{ml}$ , ●; 50  $\mu\text{g}/\text{ml}$ , ○) or PPD (10  $\mu\text{g}/\text{ml}$ , ▲; 50  $\mu\text{g}/\text{ml}$ , △) for 5 d. (C) Effect on mitogen-induced T cell proliferation. Nylon wool-purified splenic T cells ( $10^5$ ) from BALB/c mice were cultured with Con A (1.0  $\mu\text{g}/\text{ml}$ , ●; 5  $\mu\text{g}/\text{ml}$ , ○) or PHA (1  $\mu\text{g}/\text{ml}$ , ▲) for 72 h. In all experiments D7 was added at culture initiation at the indicated concentration.  $[^3\text{H}]\text{TdR}$  incorporation was measured during the last 16 h of the culture. Proliferation of unstimulated T cells was always  $<1,000$  cpm.

TABLE V  
Induction of T Cell Activation by Anti-Ly-6 mAbs

| mAb      | Specificity      | $\Delta$ cpm $[^3\text{H}]\text{TdR}$ incorporation |         |        |
|----------|------------------|---|---------|--------|
|          |                  | Exp. 1  | Exp. 2  | Exp. 3 |
| D7       | Ly-6A.2 + E.1    | 88,366  | 140,899 | 88,904 |
| S8.106   | Ly-6A.2          | 61,035  | 160,477 | 60,259 |
| 34-11-3  | Ly-6A.2          | 35,911  | 93,537  | 61,037 |
| 109-9-17 | Ly-6C.2          | 42,115  | ND      | 25,856 |
| 6C3      | Ly-6C            | ND  | 36,171  | 15,074 |
| M1/9.3.4 | Common leukocyte | 3,258   | 1,576   | 0      |

Nylon column-passed, anti-Ia and C-treated, C57BL/6 T cells ( $5 \times 10^6$ ) were incubated with the designated mAb (ascites, 1/200) for 1 h at  $4^\circ\text{C}$ , washed, and then cultured ( $2 \times 10^5$  cells/well) for 72 h in the presence of PMA (10 ng/ml) and rabbit anti-rat Ig serum (1/1000) for 72 h. Results are expressed as  $\Delta$  cpm  $[^3\text{H}]\text{TdR}$  incorporation [(cpm in the presence of PMA and anti-Ig) - (cpm in the presence of PMA alone)]. The background responses of T cells that were not preincubated with an mAb, but were cultured in the presence of PMA and the anti-Ig serum, were uniformly  $<4,000$  cpm.

characterized anti-Ly-6 mAbs for their capacity to induce T cell activation (Table V). C57BL/6 T cells were therefore pretreated with a number of different anti-Ly-6 reagents, washed, and then cultured in the presence of PMA and a crosslinking anti-Ig reagent. Although differences in the potencies of the different mAbs were observed, all could induce significant proliferative responses. In contrast to the results obtained with D7 where Fc receptor-bearing T cell-depleted spleen cells could function as effective crosslinkers, proliferative responses to all the other mAbs in Table V were only observed when anti-Ig reagents were used as the crosslinker (data not shown).



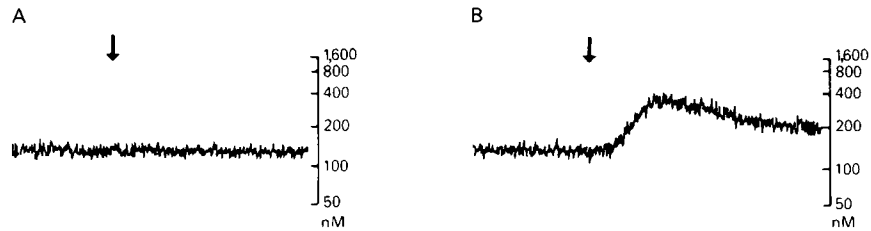


FIGURE 4. Tracing  $[Ca^{2+}]_i$  in T cell hybridoma 2B4. The ordinate on the right side of the panel shows the absolute levels of  $[Ca^{2+}]_i$ . (A) Addition of goat anti-rat IgG. (B) Addition of goat anti-rat IgG to hybridoma cells that had been preincubated with D7.

*mAb D7 Triggers a Rapid Rise in  $[Ca^{2+}]_i$ .* It has been previously shown (5, 37) that a number of mAbs to both murine and human cell surface antigens that can induce T cell activation can also induce a rapid rise in  $[Ca^{2+}]_i$  when added to T cells. Addition of mAb D7 to 2B4 hybridoma cells failed to increase  $[Ca^{2+}]_i$  (results not shown). However, when the 2B4 hybridoma cells were incubated with mAb D7, washed, and then incubated with a goat anti-rat IgG reagent, we observed a rapid rise (162%) in  $[Ca^{2+}]_i$  (Fig. 4B); no increase in  $[Ca^{2+}]_i$  was seen when the hybridoma cells were treated with the anti-Ig reagent alone (Fig. 4A). In two other experiments of this type performed with a second T cell hybridoma, we observed 123 and 205% rises of  $[Ca^{2+}]_i$ .

### Discussion

We have shown in this report that an mAb, D7, which is specific for a framework determinant on an Ly-6 antigen, could induce T cell activation after crosslinking by anti-Ig reagents or by Fc receptor-bearing AC. The ability of mAb D7 to trigger T cell proliferation in resting murine T cells required the addition of a costimulator, PMA, while D7 could induce IL-2 production from T cell hybridomas in the absence of a costimulator. In the presence of a crosslinker and PMA, D7 was equally mitogenic for L3T4<sup>+</sup> and Ly-2<sup>+</sup> T cells. Although a subset of B lymphocytes and ~10% of normal thymocytes<sup>2</sup> also express the Ly-6 molecule defined by mAb D7, we thus far cannot induce proliferative responses in these cell populations under a variety of experimental conditions (data not shown). It was somewhat surprising that D7, in the absence of PMA, could augment antigen- and alloantigen-, but not mitogen-induced T cell proliferation. One explanation for this result is that the interaction of D7 with Ly-6 caused an increase in IL-2 secretion, resulting in enhanced proliferation; the capacity of D7 to directly induce IL-2 secretion in T cell hybridomas is consistent with this interpretation. However, if this explanation accounted for augmentation of normal T cell responses, D7 should have also enhanced mitogen-induced proliferation, because other studies have shown that the addition of exogenous IL-2 to cultures of T cells stimulated with Con A or PHA, especially at suboptimal mitogen concentrations, invariably led to enhanced proliferative responses (unpublished data). An alternative explanation is that the Ly-6 molecule may function to amplify weak signals transmitted by antigen or alloantigen triggering of the T3-Ti complex, while mitogen-induced triggering may be operating optimally through the Ly-6 pathway, thus accounting for the failure

of D7 to augment mitogen-induced proliferative responses. We also cannot exclude the possibility that T cell stimulation by antigen or alloantigen, but not mitogen, somehow facilitates the response of nonantigen specific T cells to stimulation by mAb D7.

The ability to activate T cells via Ly-6 was not a property unique to mAb D7; several other mAbs to Ly-6 molecules also stimulated proliferation of resting T cells after crosslinking and in the presence of PMA. It should be emphasized that mAbs to both Ly-6A.2 and Ly-6C.2 determinants were mitogenic, and that these mAbs identify determinants with markedly disparate tissue distributions (17, 18). We have previously shown that the binding of mAb D7 to cells from Ly-6.1<sup>+</sup> strains can be completely blocked by mAb HD-42.<sup>2</sup> It is noteworthy that Flood et al. (38) have recently implicated Ly-6 molecules in T cell function by virtue of the ability of HD-42 to inhibit a number of T cells responses *in vitro*, including IL-2-dependent proliferation of mitogen-activated T cells blasts. We have thus far been unable to repeat the studies of Flood et al. (38) with mAb HD-42 and, in fact, in preliminary experiments both mAb HD-42 and a second mAb to Ly-6E.1, SK70.94, both appear to induce T cell activation after crosslinking by anti-Ig and the addition of PMA in a manner identical to that seen with the other anti-Ly-6 mAbs (Table V). The discrepancies between our results and those of Flood et al. are presently under study.

There are a number of similarities between the induction of T cell activation via Ly-6 and the induction of activation via T3-Ti, T11, and Thy-1. Activation through all of these cell surface molecules usually results in a rapid rise in  $[Ca^{2+}]_i$ , which is subsequently followed by IL-2-dependent proliferative responses (5-7, 37). Activation via T3-Ti, Thy-1, and Ly-6 all require crosslinking of the stimulating mAb after it binds to its target antigen. Although stimulation via T11 is AC-independent and does not exhibit any obvious requirement for a second crosslinking antibody (37), activation via T11 requires the simultaneous presence of two mAbs to distinct epitopes on this molecule, which may in concert lead to aggregation of T11 molecules in a manner similar to that achieved by crosslinking the mAbs to the other cell surface antigens. Despite these similarities in the requirements for activation by these mAbs there is no obvious biochemical relationship between the 14-18 kD molecules recognized by anti-Ly-6 mAbs and the components of the T3-Ti complex, T11 or Thy-1.

Rock et al. (13) have recently identified an mAb to a novel 10-12 kD T cell membrane antigen, TAP, which in soluble form will induce IL-2 production from T cell hybridomas and induce proliferation of normal T cells in the presence of AC or purified IL-1. Anti-TAP mAbs in suboptimal concentration could also augment antigen-specific T cell proliferation. The antigen identified by anti-TAP, a 10-12 kD band on SDS-PAGE under nonreducing conditions, appears to be distinct from the 14-18 kD antigen identified by most anti-Ly-6 mAbs, including D7 (19, 39).<sup>2</sup> However, TAP and Ly-6 determinants were coexpressed on all cell types examined, TAP expression mapped to the Ly-6 locus (39), and the binding of anti-TAP could be blocked by an anti-Ly-6 mAb (13). One intriguing possibility is that TAP and Ly-6 are associated with each other in a complex similar to that described for T3. Thus, mAbs directed to different

components of the complex may have different requirements for triggering or even inhibiting the T cell activation cascade.

The physiological role of the Ly-6 molecule in T cell activation has not been established in this study. Activation via Ly-6 might be postulated to occur by either of two general models. In one, Ly-6 would function as a receptor for a ligand that activates T cells upon binding Ly-6. In such a model, anti-Ly-6 mAbs would serve to mimic the physiological action of such a ligand. This would be analogous to the situation where anti-Ig, or anti-T cell receptor antibodies, or some anti-hormone receptor antibodies activate cellular responses. The ligand for Ly-6 might be a lymphokine or a distinct cell surface antigen, perhaps expressed on the cell surface of an APC. In this model, Ly-6 may function as an alternative pathway for activation of murine T cells in a manner similar to that suggested for T11 (7, 14). In a second model, Ly-6 could function solely as a signal-transducing molecule with no external ligand. Ly-6 might then stabilize, amplify, or transduce signals originally initiated by antigen-stimulation of the T3-Ti complex. Ly-6 could activate T cell function either alone or as a member of a larger molecular complex. In view of the general difficulty we and others have noted in radiolabeling and immunoprecipitating Ly-6 molecules (17, 19), it is even possible that it could be found in association with the T3-Ti complex or Thy-1. Future study is necessary to resolve these issues.

### Summary

The Ly-6 locus controls the expression and/or encodes for alloantigenic specificities found primarily on subpopulations of murine T and B lymphocytes. We have recently identified and characterized a new rat mAb, D7, that recognizes a nonpolymorphic Ly-6 specificity. After crosslinking by anti-Ig reagents or by Fc receptor-bearing accessory cells, mAb D7 could induce IL-2 production from T cell hybridomas, and in the presence of PMA could trigger a vigorous proliferative response in resting peripheral T cells. The addition of mAb D7 to cultures of antigen- and alloantigen-, but not mitogen-stimulated T cells resulted in a marked augmentation of the proliferative response. A number of other well-characterized mAbs to Ly-6 locus products could also stimulate a T cell proliferative response after crosslinking by anti-Ig and in the presence of PMA. These results strongly suggest that Ly-6 molecules may play a critical role in the T cell activation cascade, either as receptors for an unidentified soluble or cell-associated ligand or as transducing molecules that modulate signals initiated by antigen stimulation of the T3-Ti complex.

We thank Dr. Jeffrey Bluestone for helpful advice and careful review of the manuscript. We also appreciate the expert assistance of Ms. Shirley Starnes in the preparation of this manuscript.

*Received for publication 30 April 1986.*

### References

1. Reinherz, E. L., O. Acuto, M. Fabbi, A. Bensussan, C. Milanese, H. D. Royer, S. C. Meuer, and S. F. Schlossman. 1984. Clonotypic surface structure on human T

- lymphocytes: functional and biochemical analysis of the antigen receptor complex. *Immunol. Rev.* 81:95.
2. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med.* 157:1149.
  3. Samelson, L. E., J. B. Harford, and R. D. Klausner. 1985. Identification of the components of the murine T cell antigen receptor complex. *Cell.* 43:223.
  4. Oettgen, H. C., C. L. Pettey, W. L. Maloy, and C. Terhorst. 1986. A T3-like protein complex associated with the antigen receptor on murine T cells. *Nature (Lond.)* 320:272.
  5. Weiss, A., J. Imboden, D. Shoback, and J. D. Stobo. 1984. Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA.* 81:4169.
  6. Weiss, A. and J. D. Stobo. 1984. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* 160:1284.
  7. Meuer, S. C., R. E. Hussey, M. Fabbi, D. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgdon, J. P. Protentis, S. F. Schlossman, and E. I. Reinherz. 1984. An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. *Cell.* 36:897.
  8. Hara, T., S. M. Fu, and J. A. Hansen. 1985. Human T cell activation. II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44 kilodalton polypeptide (9.3 antigen). *J. Exp. Med.* 161:1513.
  9. Moretta, A., G. Pantaleo, M. Lopez-Botet, and L. Moretta. 1985. Involvement of T44 molecules in an antigen-independent pathway of T cell activation. Analysis of the correlations to the T cell antigen-receptor complex. *J. Exp. Med.* 162:823.
  10. Smith, R. T., S. Shimizu, M. A. Norcross, and V. C. Maino. 1982. Mechanism of TCGF production by cloned T-cell lines: characterization of producer lymphoma lines and kinetics of production. *Clin. Immunol. Immunopathol.* 23:286.
  11. Gunter, K. C., T. R. Malek, and E. M. Shevach. 1984. T cell-activating properties of an anti-Thy-1 monoclonal antibody: possible analogy to OKT3/LEU-4. *J. Exp. Med.* 159:716.
  12. MacDonald, H. R., C. Bron, M. Rousseaux, C. Horvath, and J.-C. Cerottini. 1985. Production and characterization of monoclonal anti-Thy-1 antibodies that stimulate lymphokine production by cytolytic T cell clones. *Eur. J. Immunol.* 15:495.
  13. Rock, K. L., E. T. H. Yeh, C. F. Gramm, S. I. Haber, H. Reiser, and B. Benacerraf. 1986. TAP, a novel T cell-activating protein involved in the stimulation of MHC-restricted T lymphocytes. *J. Exp. Med.* 163:315.
  14. Fox, D. A., R. E. Hussey, K. A. Fitzgerald, A. Bensussan, J. F. Daley, S. F. Schlossman, and E. L. Reinherz. 1985. Activation of human thymocytes via the 50 kd T11 sheep erythrocyte binding protein induces the expression of interleukin 2 receptors on both T3<sup>+</sup> and T3<sup>-</sup> populations. *J. Immunol.* 134:330.
  15. Meruelo, D., M. Offer, and A. Rossmando. 1982. Evidence for a major cluster of lymphocyte differentiation antigens on murine chromosome 2. *Proc. Natl. Acad. Sci. USA.* 79:7460.
  16. Kimura, S., N. Tada, E. Nakayama, and U. Hammerling. 1980. Studies on the mouse Ly-6 alloantigen system. I. Serological characterization of mouse Ly-6 alloantigen by monoclonal antibodies. *Immunogenetics.* 11:373.
  17. Auchincloss, H., K. Ozato, and D. H. Sachs. 1981. Two distinct murine differentiation antigens determined by genes linked to the Ly-6 locus. *J. Immunol.* 127:1839.
  18. Kimura, S., N. Tada, Y. Liu-Lam, and U. Hammerling. 1984. Studies of the mouse Ly-6 alloantigen system. II. Complexities of the Ly-6 region. *Immunogenetics.* 20:47.

19. Palfree, R. G. E. and U. Hammerling. 1986. Biochemical characterization of the murine activated lymphocyte alloantigen Ly-6E.1 controlled by the Ly-6 locus. *J. Immunol.* 136:594.
20. Dumont, F. J., L. Z. Coker, R. C. Habbersett, and J. A. Treffinger. 1985. Xenogeneic monoclonal antibody to an Ly-6-linked murine cell surface antigen: differential reactivity with T cell subpopulations and bone marrow cells. *J. Immunol.* 134:2357.
21. Marshak-Rothstein, A., P. Fink, T. Gridley, D. H. Raulet, M. J. Bevan, and M. L. Gefter. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* 122:2491.
22. Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Harvran, 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.
23. Hammerling, G. J., U. Hammerling, and L. Flaherty. 1979. Qat-4 and Qat-5, new murine T-cell antigens governed by the T1a region and identified by monoclonal antibodies. *J. Exp. Med.* 150:108.
24. Sarmiento, M., D. P. Dialynas, D. W. Lancki, K. A. Wall, M. I. Lorber, M. R. Loken, and F. W. Fitch. 1982. Cloned T lymphocytes and monoclonal antibodies as probes for cell surface molecules active in T cell-mediated cytotoxicity. *Immunol. Rev.* 68:135.
25. Bhattacharya, A., M. E. Dorf, and T. A. Springer. 1981. A shared alloantigenic determinant on Ia antigen encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* 127:2488.
26. Malek, T. R., T. J. Robb, and E. M. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA.* 80:5694.
27. Ortega, R. G., R. J. Robb, E. M. Shevach, and T. R. Malek. 1984. The murine IL 2 receptor. I. Monoclonal antibodies that define distinct functional epitopes on activated T cells and react with activated B cells. *J. Immunol.* 133:1970.
28. Springer, T. 1980. Cell-surface differentiation in the mouse. In *Monoclonal Antibodies*. R. Kenneth, T. J. McKearn, and K. B. Bechtol, editors. Plenum Press, New York. 185.
29. Springer, T., G. Galfre, D. S. Secker, and C. Milstein. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.* 8:539.
30. Unkless, J. C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptor. *J. Exp. Med.* 150:580.
31. Lanier, L. L., G. A. Gutman, D. E. Lewis, S. T. Griswold, and N. L. Warner. 1982. Monoclonal antibodies against rat immunoglobulin chains. *Hybridoma.* 1:125.
32. Glimcher, L. H. and E. M. Shevach. 1982. Production of autoreactive I region-restricted T cell hybridomas. *J. Exp. Med.* 156:640.
33. Malek, T. R., J. A. Schmidt, and E. M. Shevach. 1985. The murine IL 2 receptor. III. Cellular requirements for the induction of IL 2 receptor expression on T cell populations. *J. Immunol.* 134:2405.
34. Kappler, J., J. White, D. Wegmann, E. Mustain, and P. Marrack. 1982. Antigen presentation by Ia<sup>+</sup> B cell hybridomas to H-2 restricted T cell hybridomas. *Proc. Natl. Acad. Sci. USA.* 79:3604.
35. Tsien, R. Y., T. Pozzan, and T. J. Rink. 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell. Biol.* 94:325.
36. Tsien, R. Y., T. Pozzan, and T. J. Rink. 1982. T-cell mitogens cause early changes

in cytoplasmic free  $\text{Ca}^{2+}$  and membrane potential in lymphocytes. *Nature (Lond.)*. 295:68.

37. Kroczek, R. A., K. C. Gunter, B. Seligman, and E. M. Shevach. 1986. Induction of T cell activation by monoclonal anti-Thy-1 antibodies. *J. Immunol.* 136:4379.
38. Flood, P. M., D. B. Murphy, F. R. Smith, E. Stockert, M. A. Palladino, Jr., and A. B. DeLeo. 1985. A monoclonal antibody that recognizes an Ly-6-linked antigen inhibits the generation of functionally active T cell subsets. *J. Immunol.* 135:63.
39. Reiser, H., E. T. H. Yeh, C. F. Gramm, B. Benacerraf, and K. L. Rock. 1986. The genes encoding TAP and TAPa map to the Ly-6 locus. *Proc. Natl. Acad. Sci. USA.* 83:2954.