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T D Geppert, P E Lipsky

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Research Article

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Activation of T Lymphocytes by Immobilized Monoclonal Antibodies to CD3

Regulatory Influences of Monoclonal Antibodies to Additional T Cell Surface Determinants

Thomas D. Geppert and Peter E. Lipsky
Harold C. Simmons Arthritis Research Center, Department of Internal Medicine, University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, Texas 75235

Abstract

The effect of soluble or immobilized MAb directed at various additional surface proteins on the proliferation of highly purified T_4 cells induced by two immobilized MAb to CD3, OKT3 and 64.1, was examined. High density 64.1 stimulated nearly all T_4 cells to enter and progress through the cell cycle. Maximal T_4 cell proliferation required stimulation with immobilized 64.1 throughout the length of the incubation and was not effected by any of the additional soluble or immobilized MAb employed. In contrast, low density immobilized 64.1 and all densities of immobilized OKT3 employed stimulated a minority of the cells to enter the cell cycle and proliferate. Immobilized MAb directed at CD2, class I major histocompatibility complex (MHC) encoded gene products or CD11a (LFA-1) dramatically enhanced the response, whereas soluble MAb directed at these determinants did not. Both immobilized and soluble MAb directed at CD5 and CD28 (Tp44) enhanced responses, but they were less effective than immobilized MAb to CD2, LFA-1 or HLA-A,B,C. Soluble anti-CD4 MAb inhibited responses somewhat, whereas immobilized anti-CD4 enhanced responses. Costimulation was observed when MAb to CD3 and class I MHC molecules but not CD2, LFA-1 or CD4 were immobilized to separate surfaces. The data suggest that when anti-CD3 stimulation is suboptimal, responses can be enhanced by MAb to CD5 or CD28 (Tp44) or by immobilized MAb to CD4, CD2, CD11a (LFA-1), or class I MHC encoded gene products. Although crosslinking of CD4, CD2, or CD11a with CD3 may be necessary for costimulation, immobilized MAb to CD3 and class I MHC molecules appear to deliver independent signals that result in enhanced T_4 cell activation and proliferation.

Introduction

Soluble monoclonal antibodies (MAb) directed at CD3 induce accessory cell (AC)[1] dependent T cell proliferation (1, 2). One role of the AC is to bind the anti-CD3 MAb by Fc receptors and thus create a matrix to cross-link anti-CD3 bound CD3 molecules (3, 4). In addition, AC promote anti-CD3 induced T cell proliferation through AC-T cell interactions involving a variety of AC and T cell surface proteins including CD11a (LFA-1), and class I and II major histocompatibility complex (MHC) encoded gene products (5). The exact role of these interactions is not completely understood. One possibility, however, is that the AC provides a cellular matrix to cross-link a variety of T cell surface molecules and that cross-linking these surface molecules together with the CD3 complex promotes T cell activation.

In contrast to the AC requirement for soluble anti-CD3 induced T cell activation, T_4 cell proliferation can be stimulated in the complete absence of AC when the anti-CD3 is immobilized onto a polystyrene surface (6). The response is directly related to the density and the nature of the immobilized anti-CD3. Thus, a high density of one of the anti-CD3 MAb, 64.1, induces vigorous proliferation that is not enhanced by accessory cell signals, whereas lower densities of 64.1 induce suboptimal T_4 cell responses that can be enhanced by auxiliary influences. Responses induced by still lower densities of 64.1 are completely dependent on additional signals (6). These findings suggest a model wherein maximal anti-CD3 induced T_4 cell activation requires multiple interactions with CD3. When the stimulus is suboptimal, responses are dependent on signals provided by AC or MAb that deliver AC like signals. The mechanism whereby AC promote responses induced by suboptimal stimulae is unclear.

These studies were undertaken to determine whether immobilized MAb to other T_4 cell surface molecules might simulate some AC-T cell interactions and enhance T_4 cell proliferation induced by suboptimal stimulation via the CD3 molecule. To investigate this possibility, highly purified T_4 cells depleted of all AC were used as the responding cells and the effects of immobilized and soluble MAb directed at various T cell surface molecules including CD11a (LFA-1), CD2, CD4, CD5, CD28 (Tp44), and class I MHC encoded gene products were examined. MAb directed at these antigens were chosen because it has been suggested that these determinants may be involved in accessory cell-T cell interactions (5, 7–12). The effect of these MAb on suboptimal stimulation by immobilized anti-CD3 can be divided as follows. One group of MAb had minimal effects on immobilized anti-CD3 induced proliferation in the soluble form, but enhanced proliferation markedly when immobilized. This group includes MAb directed at CD2, CD11a (LFA-1), and class I MHC encoded gene products. MAb to class I MHC molecules were unique in that they could facilitate immobilized anti-CD3 induced T_4 cell proliferation even when presented on a separate solid matrix. The second group, made up of MAb to CD28 (Tp44) and CD5, enhanced responses in the soluble form; immobilization did not increase their ability to enhance responses. The third effect noted occurred with MAb to CD4 that inhibited responses in

Address reprint requests to Dr. Lipsky, Department of Internal Medicine, UTHSCD, Southwestern Medical School, 5323 Harry Hines Boulevard, Dallas, TX 75235.

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1. *Abbreviations used in this paper:* AC, accessory cell; AO, acridine orange; APC, antigen presenting cell; GaMIg, goat anti-mouse immunoglobulin; MHC, major histocompatibility complex; Mø, monocytes; NHS, normal human serum.

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the soluble form, but enhanced responses when immobilized. The data indicate that anti-CD3 induced T_4 cell activation may be modulated by MAb to other T_4 cell surface molecules. The effect of these MAb is most noteworthy when the strength of the activation signal delivered via CD3 is suboptimal and varies with the particular cell surface molecule recognized by the additional MAb and whether the MAb is soluble or immobilized. The results indicate that many of the signals normally delivered to T_4 cells by accessory cells can be simulated by a variety of soluble and immobilized MAb to specific T_4 cell surface determinants.

Methods

Monoclonal antibodies. A variety of T cell specific MAb were used including OKT3 (Ortho Pharmaceutical Corp., Raritan, NJ) and 64.1 (gift from Dr. Ellen Vitetta), IgG2a MAb directed at the CD3 molecule on mature T cells (13, 14), 9.6 (a generous gift from Dr. Patrick Beatty, Fred Hutchinson Cancer Center, Seattle, WA) and OKT11 (American Type Culture Collection [ATCC]), IgG2a MAb directed at the E rosette receptor found on T cells (15, 16), 66.1 (gift from Dr. Patrick Beatty), and G17-2 (gift of Dr. Jeffrey Ledbetter, Oncogene, Seattle, WA), IgM and IgG1 MAb, respectively, directed at CD4 (13), 9.3 (gift of Dr. Jeffrey Ledbetter), a MAb directed at a 44 kD homodimer (Tp44, CD28) found on the surface of most T cells (17), 60.3 (gift from Dr. Patrick Beatty), an IgG2a MAb directed at an epitope common to LFA-1 (CD11a), CD11b and the p150.95 molecule (7), 10.2 (gift of Dr. Jeffrey Ledbetter), an IgG2a MAb directed at the CD5 T cell surface molecule (18). In addition, several MAb directed at class I encoded gene products including PA2.6 and W6/32 (19, 20), IgG2a MAb, and MB40.5 (19), an IgG1 MAb and a variety of miscellaneous MAb including MOPC (ATCC) and P1.17 (ATCC), control IgG1 and IgG2a MAb, respectively, and HNK-1 (ATCC), an NK cell specific IgM MAb (21) were employed. The control IgG2a MAb was purified by passage over a Sephacryl S-300 column, dialyzed, and filter sterilized before use. 10.2, G17-2, 60.3, 9.3, 64.1, OKT11, 9.6, PA2.6, MB40.5, and W6/32 were purified over a column of Sepharose 4B coupled with staphylococcal protein A. 66.1 and HNK-1 ascites were diluted and added directly to the wells at a 1:5,000 final dilution.

Reagents. Recombinant IL-2 was kindly provided by Cetus Corp., Emeryville, CA.

Cell preparation. Peripheral blood mononuclear cells (PBM) were obtained from healthy adult volunteers by centrifugation of heparinized venous blood over sodium diatrizoate/Ficoll gradients (Isolymph; Gallard Schlessinger Chemical Mfg. Corp., Carle Place, NY). Cells were washed three times in HBSS before further processing.

T cell purification. T cells were prepared from PBM as previously described (22). Briefly, PBM were incubated with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co., St. Louis, MO) to remove contaminating Mϕ (23). After washing twice in HBSS, cells forming rosettes with neuraminidase-treated sheep red blood cells were isolated and passed over a nylon wool column to deplete residual B cells and Mϕ.

Purified T_4 cells were prepared by negative selection using a panning technique (24) to deplete contaminating Ia positive cells and T_8 cells. Cells were reacted with saturating concentrations of the anti-Ia MAbs, IVA12 and L243 plus OKT8. After washing, the cells were added to goat antimouse immunoglobulin (GaMIg) coated panning dishes and incubated for 90 min at 4°C. Afterward the nonadherent cells were gently aspirated and panned a second time on another GaMIg-coated petri dish. The nonadherent T_4 cells were harvested and found to contain $< 0.1\%$ esterase positive cells, $< 3\%$ T_8 positive cells and $> 96\%$ T_4 positive cells. Viability of the T_4 cell population was always $> 95\%$.

Adherence of MAb to the culture wells or polystyrene beads. MAb were immobilized onto plastic microtiter plates as described (6). Briefly, 50 μl of MAb, diluted in RPMI, were placed in each of the wells of 96-well flat-bottomed microtiter plates (No. 3799; Costar, Cambridge, MA) and incubated at room temperature for 1 h, and then washed with saline one to three times to remove nonadherent MAb. When an additional MAb was immobilized to the same wells, the process was repeated with the additional MAb. Quantitation of adherent MAb was determined using bicinchoninic acid and $CuSO_4$ at pH 11.25 as described (6). When 3 μg/well or less MAb were incubated in microtiter wells as described, 15–20% of the added material adhered to the well. The adherence of the various MAb to the wells was comparable. OKT3 was immobilized onto polystyrene beads (Dupont de Nemours, Wilmington, DE) by suspending the 2.26-μM beads in Tris buffer, pH 9.5, containing OKT3 (100 μg/ml). The beads were incubated with OKT3 for 120 min at 4°C and then washed three times. The beads were then resuspended in culture medium and added to the wells. The density of OKT3-coated beads utilized completely covered the bottom of the microtiter wells.

Techniques of cell culture and assay of T_4 cell DNA synthesis. Cells were cultured in medium RPMI 1640 supplemented with 10% heat-inactivated normal human serum (NHS), penicillin G (200 U/ml), gentamicin (10 μg/ml), and L-glutamine (0.3 mg/ml). Most cultures were carried out in 96-well microtiter plates with flat-bottomed wells in a total volume of 200 μl. 1×10^5 T_4 cells were cultured in MAb containing wells with or without additional immobilized or soluble MAb. IL-2 (10 U/ml) was added where indicated. Soluble MAb were added after the addition of human serum containing medium. 18 h before the cells were harvested by using a semiautomated cell harvester (Microbiological Associates, Walkersville, MD), 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to the cultures. [^{3}H]Thymidine incorporation was measured in a liquid scintillation counter. All data are expressed as the mean counts per minute of triplicate determinations.

Removal of T_4 cells from wells containing immobilized MAb. To remove T_4 cells from wells containing immobilized MAb, the cells were harvested by gently pipetting the medium in and out of the well, aspirating the cells and washing them once with saline. Remaining cells were detached by adding PBS containing 5 mM EDTA, 0.5% glucose, and 0.1% trypsin for 3 min. These cells were then added to the cells removed with pipetting alone, centrifuged, washed, and suspended in medium.

RNA and DNA content. Cellular content of RNA and DNA was determined after acridine orange (AO) staining using the technique described by Darzynkiewicz et al. (25). Briefly, 3×10^5 cells per sample were collected from cultures in microtiter wells after 30 h of incubation, washed once and suspended in fresh medium containing 10% FBS. The cells were made permeable in a chilled solution containing 0.1% (vol/vol) Triton X-100 (Sigma Chemical Co.), 0.2 M sucrose, 10^{-4} M EDTA, and 2×10^{-2} M citrate phosphate buffer, at pH 3.0. After 1 min, the cells were stained by adding a second solution containing 0.002% purified AO (Polysciences, Inc., Warrington, PA), 0.1 M NaCl, and 10^{-2} M citrate phosphate buffer, at pH 3.8. The cells were analyzed with a cytofluorograph (System 50-HH; Ortho Diagnostic Instruments, Westwood, MA) using an argon laser line setting of 488 nm at 50 mW. The red (RNA) and green (DNA) fluorescence emissions from each cell were separated and quantitated by individual photomultipliers. The cytofluorograph was interfaced to a Data General (Milford, MA) 2150 computer for analysis. Dead cells and cell doublets were excluded using a previously described method (26). The data are based on analysis of a total of 5×10^3 cells per sample unless otherwise stated.

Results

The effect of various MAb directed at T_4 cell surface molecules on immobilized OKT3 induced T_4 cell proliferation. The initial experiments utilized a suboptimal concentration of OKT3 to stimulate T cells to determine whether the presence of additional MAb to T cell surface molecules could amplify DNA

synthesis. The effect of soluble or immobilized MAb directed at various non-CD3 T_4 cell surface proteins was examined. As shown in Fig. 1, low density immobilized OKT3 induced minimal proliferation of AC-depleted T_4 cells. The response was not effected by soluble control MAb but was markedly enhanced by soluble 9.3 (anti-CD28). Soluble 60.3 (anti-LFA-1), MB40.5 (anti-HLA-A,B,C), and OKT11 (anti-CD2) did not enhance or inhibit the response. When immobilized, the MAb to LFA-1, CD2 and HLA-A,B,C dramatically enhanced T_4 cell proliferation induced by immobilized OKT3. Other anti-HLA-A,B,C MAb, including W6/32 and PA2.6 and the anti-CD2 MAb, 9.6, gave similar results (data not shown). Maximal proliferation was comparable when MAb to CD2, LFA-1 or class I MHC molecules were employed. However, to obtain comparable enhancement of immobilized OKT3 induced responses, 10-fold greater concentrations of MB40.5 (anti-HLA-A,B,C) than 60.3 (anti-LFA-1) or OKT11 (anti-CD2) were required. In the absence of anti-CD3, none of the immobilized MAb induced T_4 cell proliferation (6). Immobilized 9.3 also enhanced the response, but was not a more effective promoter of immobilized OKT3 induced T_4 cell proliferation than soluble 9.3. Moreover, immobilized 9.3 was a less effective promoter of immobilized OKT3 induced T_4 cell proliferation than immobilized 60.3, MB40.5, or OKT11. Comparable results were noted when 10.2, a MAb to CD5, was examined. Thus, immobilized or soluble 10.2 enhanced OKT3-induced proliferation, albeit not as effectively as immobilized or soluble 9.3 (data not shown).

Immobilized and soluble MAb to CD4 have contrasting effects on immobilized OKT3 induced T_4 cell proliferation. The effect of soluble and immobilized MAb to CD4 on immobilized OKT3 induced T_4 cell proliferation was also examined. As previously reported (5), soluble MAb to CD4 inhibit immobilized OKT3-induced T_4 cell proliferation (Table I). As noted previously, soluble MAb to CD2 did not inhibit immobilized OKT3 induced responses. When the additional MAb were immobilized, however, both anti-CD2 and anti-CD4 enhanced responses. Immobilized MAb to CD4, however, enhanced responses somewhat less effectively than immobilized MAb to CD2. Similar results were obtained when the amplifying capacity of anti-CD4 was compared to that of anti-HLA-A,B,C or anti-LFA-1 (data not shown).

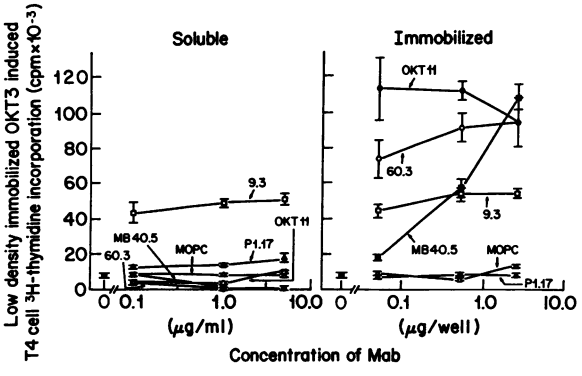

Figure 1. The effect of various MAb directed at T_4 cell surface molecules on low density immobilized OKT3 induced T_4 cell proliferation. T_4 cells (1×10^5) were cultured with immobilized OKT3 (20 ng/well) plus additional immobilized or soluble OKT11 (anti-CD2), 60.3 (anti-LFA-1), MB40.5 (anti-HLA-A,B,C), 9.3 (anti-CD28), or P1.17, a control MAb, as indicated. After 3 d in culture, [^{3}H]thymidine incorporation was assessed and the data are expressed as the mean counts per minute of triplicate determinations±SEM. T_4 cells stimulated by immobilized OKT3 in the absence of additional MAb incorporated 6,400±700 cpm.

Table I. Immobilized and Soluble Anti-CD4 Antibodies Have Contrasting Effects on Immobilized OKT3-induced Proliferation

Experiment	Additional monoclonal antibody: Form	Designation	Specificity	OKT3 induced T_4 cell DNA synthesis
				[^{3}H]Thymidine incorporation (cpm × 10^{-3})
1	Soluble	HNK-1	NK cells	18.2±1.6
	Soluble	66.1	CD4	1.9±0.1
	Immobilized	HNK-1	NK cells	11.1±1.6
	Immobilized	66.1	CD4	50.7±0.9
2	Soluble	MOPC	Control	35.0±0.4
	Soluble	G17-2	CD4	12.0±1.4
	Soluble	OKT11	CD2	34.5±1.7
	Immobilized	MOPC	Control	29.6±1.6
	Immobilized	G17-2	CD4	52.3±4.0
	Immobilized	OKT11	CD2	81.9±3.1

T_4 cells (10^5) were cultured with immobilized OKT3 (200 ng/well) and 2,000 ng of the additional MAb. [^{3}H]Thymidine incorporation was determined after 3 d in culture. The data are expressed as the mean counts per minute of triplicate determination±SEM.

Immobilized MAb directed at CD2, LFA-1 or HLA-A,B,C enhance suboptimal but not maximal proliferation induced by anti-CD3. As previously noted (6), high density immobilized 64.1 stimulates all T_4 cells to enter the cell cycle, whereas, low density immobilized 64.1 and all densities of OKT3 stimulate submaximal responses. Table II compares the magnitude of responses induced by various concentrations of OKT3 and 64.1 in the presence or absence of the additional immobilized MAb. In the presence of immobilized control MAb, immobilized OKT3 is a markedly less effective stimulator of T_4 cell DNA synthesis than immobilized 64.1 as previously noted (6). Immobilized MAb to HLA-A,B,C, LFA-1 and CD2 enhanced responses induced by all the densities of immobilized OKT3 employed, whereas they enhanced immobilized 64.1 induced responses only when suboptimal densities of this anti-CD3 were utilized. Immobilized MAb to CD5, 10.2, enhanced suboptimal responses induced by immobilized OKT3, but was less effective than MAb to CD2, LFA-1 or HLA-A,B,C. In experiments not shown, it was demonstrated that immobilized MAb to CD2, LFA-1 and HLA-A,B,C also enhanced IL-2 production stimulated by immobilized OKT3 and suboptimal concentrations of 64.1, but had no effect on the production of IL-2 induced by high concentrations of 64.1.

Immobilized MAb directed at HLA-A,B,C, LFA-1, or CD2 increase the percentage of T_4 cells induced by immobilized anti-CD3 to enter the cell cycle. To determine whether immobilized antibodies to the additional T_4 cell determinants increased the number of cells entering the cell cycle or facilitated DNA synthesis by anti-CD3 stimulated cells, acridine orange analysis was carried out. Table III depicts the effect of immobilized W6/32 on immobilized OKT3 and 64.1 induced T_4 cell cycle entry and progression. Immobilized OKT3 induced substantially fewer cells to enter the cell cycle than comparable

Table II. T_4 Cell Proliferation Induced by Immobilized OKT3 and 64.1 Is Comparable When Immobilized Anti-T Cell Antibodies Are Present

Experiment	Immobilized Anti-CD3	Immobilized monoclonal antibody				
		P1.17	60.3	9.6	W6/32	10.2
	ng/well	*Anti-CD3 MAb induced T_4 cell [^{3}H]thymidine incorporation (cpm × 10^{-3})*				
1	OKT3 (20)	0.3±0.1	65.2±4.0	89.5±1.3	75.6±10.6	ND
	64.1 (20)	54.4±6.5	114.0±7.8	100.0±5.2	97.1±12.8	ND
	OKT3 (200)	24.9±2.4	101.5±11.5	110.1±4.3	110.4±3.1	ND
	64.1 (200)	97.1±7.1	117.4±11.9	108.4±4.7	99.1±11.4	ND
2	OKT3 (7)	0.4±0.1	124.9±9.1	131.0±3.3	161.2±5.2	28.4±2.1
	OKT3 (200)	33.7±2.1	209.0±10.2	162.2±7.8	194.4±5.1	38.4±1.8
	64.1 (200)	189.9±5.9	171.3±4.2	196.7±33.4	209.1±5.9	250.2±6.4

T_4 cells were cultured with immobilized OKT3 or 64.1 at the indicated densities with additional immobilized 9.6, W6/32, 60.3, 10.2 or P1.17, a control MAb (500 ng/well). After 3 d in culture, [^{3}H]thymidine incorporation was assessed. Data are expressed as the mean counts per minute of triplicate determinations±SEM. In the absence of immobilized anti-CD3, no [^{3}H]thymidine incorporation was observed.

densities of immobilized 64.1. Immobilized W6/32 (anti-HLA-A,B,C) dramatically enhanced the number of T_4 cells induced by all densities of immobilized OKT3 to enter the cell cycle, whereas immobilized 64.1-induced cell cycle progression was enhanced only when suboptimal densities of 64.1 were employed. Immobilized W6/32 alone in the absence of an immobilized anti-CD3 MAb did not stimulate cell cycle entry of T_4 cells. Similar observations were made when immobilized 9.6 (anti-CD2) and 60.3 (anti-LFA-1) were used instead of W6/32 (data not shown).

The capacity of MAb to various T cell surface molecules to enhance immobilized anti-CD3 induced T_4 cell proliferation when the MAb to CD3 and the additional T cell surface molecules are immobilized onto different surfaces. The capacity of MAb to various T cell surface molecules to enhance anti-CD3 induced T_4 cell proliferation when the additional MAb and the anti-CD3 MAb were immobilized onto different surfaces was examined next (Table IV). Various MAb including 60.3, OKT11, G17-2, MB40.5, or MOPC as control were immobilized onto the polystyrene microtiter wells and OKT3 was immobilized onto polystyrene beads. T_4 cells did not proliferate in the presence of immobilized MOPC, 60.3, or G17-2, and

Table III. Effect of Immobilized W6/32 on the Capacity of OKT3 and 64.1 to Induce T_4 Cell Cycle Entry and Progression

Immobilized anti-CD3		Immobilized P1.17		Immobilized W6/32	
MAb	ng/well	G_1	S+G_2/M	G_1	S+G_2/M
		% of T_4 cells			
P1.17	200	1.0	1.9	0.9	1.4
64.1	200	79.4	15.7	76.0	18.3
	20	73.5	16.1	74.7	19.5
	4	7.5	1.0	30.4	4.4
OKT3	200	5.7	1.0	66.6	11.7
	20	0.3	0.0	79.3	16.1
	4	2.2	1.0	35.9	4.3

T_4 cells (1×10^5) were cultured with immobilized 64.1 or OKT3 as indicated with additional immobilized P1.17, a control MAb, or W6/32 for 30 h. The T_4 cells were then harvested, stained with AO and analyzed using a fluorescence activated cell sorter. Cells in G_1 were identified as cells with greater RNA but not DNA content than control cells. S+G_2/M was defined as those cells with both increased RNA and DNA content. The data are expressed as the percentage of T_4 cells in G_1 or S + G_2/M.

Table IV. Immobilized OKT3-induced T_4 Cell Proliferation: Effect of Monoclonal Antibodies to CD2, CD4, LFA-1, and Class I MHC Antigens Immobilized to a Separate Surface

Experiment	Immobilized to microtiter well		Immobilized to beads	
	Designation	Specificity	Nil	OKT3
			[^{3}H]thymidine incorporation (cpm × 10^{-3})	
1	MOPC	Control	0.1±0.1	0.1±0.1
	60.3	LFA-1	0.1±0.1	0.1±0.1
	MB40.5	HLA-A,B,C	2.8±0.3	14.4±1.8
	OKT11	CD2	1.3±0.1	1.1±0.2
2	MOPC	Control	0.1±0.1	0.1±0.1
	60.3	LFA-1	0.1±0.1	0.1±0.1
	MB40.5	HLA-A,B,C	0.8±0.2	9.2±1.1
	G17-2	CD4	0.1±0.1	0.1±0.1
3	MOPC	Control	0.1±0.1	0.1±0.1
	60.3	LFA-1	0.2±0.1	0.2±0.1
	MB40.5	HLA-A,B,C	1.6±0.1	8.4±1.4
	OKT11	CD2	0.1±0.1	0.2±0.1

T_4 cells (1×10^5) were cultured in microtiter wells with immobilized MOPC, 60.3, OKT11, G17-2, or MB40.5 alone or with OKT3 immobilized onto polystyrene beads. In experiment 3, responses stimulated by control beads coated with no MAb were compared to those stimulated by anti-CD3 stimulated beads. In experiment 3, [^{3}H]thymidine incorporation of T_4 cells stimulated with OKT3 immobilized to the microtiter well was 22.8±1.8 × 10^3 cpm in the presence of MOPC and 88.0±8.3, 92.6±5.4, and 50.8±0.5 × 10^3 cpm in the presence of 60.3, MB40.5 and OKT11 immobilized to the microtiter well, respectively. [^{3}H]Thymidine incorporation was determined following 3 d in culture. The data are expressed as the mean counts per minute of triplicate determinations±SEM.

Table V. Immobilized Antibodies to T Cell Surface Molecules Augment T_4 Cell Proliferation Stimulated by Immobilized but Not Soluble Anti-CD3

Experiment	Immobilized monoclonal antibody	OKT3		64.1	
		Soluble	Immobilized	Soluble	Immobilized
		[^3H]Thymidine incorporation (cpm × 10^{-3})			
1	P1.17	0.1±0.1	0.1±0.1	0.5±0.1	96.7±13.8
	9.6	0.1±0.1	85.6±6.0	0.3±0.1	60.7±6.5
	60.3	0.1±0.1	58.6±5.7	0.1±0.1	92.2±4.1
	OKT11	0.1±0.1	63.1±6.9	0.5±0.1	75.1±5.1
2	P1.17	0.1±0.1	ND	0.4±0.1	5.8±0.9
	W6/32	0.1±0.1	ND	0.4±0.1	32.5±4.6

T_4 cells were cultured with immobilized (100 ng/well in experiment 1 or 20 ng/well in experiment 2) or soluble (1 μg/ml) OKT3 or 64.1 with immobilized P1.17, a control MAb, OKT11, 9.6, 60.3, or W6/32 each at 500 ng/well. After 3 d in culture, [^{3}H]thymidine incorporation was assessed. The data are expressed as the mean counts per minute of triplicate determinations±SEM.

proliferated minimally in the presence of immobilized OKT11 or MB40.5 in the absence of immobilized OKT3. Unlike OKT3 immobilized to the microtiter wells, OKT3 immobilized to polystyrene beads did not induce T cell proliferation alone. Moreover, proliferation was not observed even in the presence of immobilized MOPC, G17-2, or 60.3. In addition, the minimal responses obtained in the presence of immobilized OKT11 and anti-CD3 were not greater than those obtained in the absence of immobilized OKT3. Increasing or decreasing the density of OKT3 coated beads did not result in responses alone or with immobilized MOPC, G17-2, 60.3, or OKT11 (data not shown). In contrast, OKT3 immobilized to beads stimulated T_4 cell DNA synthesis in the presence of MB40.5 immobilized to the microtiter plates. These results indicate that MAb to class I MHC molecules are unique in that costimulation with anti-CD3 was observed when each MAb was presented on a separate solid matrix.

Soluble MAb directed at CD3 do not induce T_4 cell proliferation in the presence of immobilized MAb directed at CD2, LFA-1, or HLA-A,B,C. The possibility that soluble anti-CD3 could induce T_4 cell proliferation in the presence of immobilized MAb directed at CD2, LFA-1, or HLA-A,B,C was examined next. As can be seen in Table V, T_4 cell proliferation was only noted in the presence of the immobilized anti-CD3 MAb.

Immobilized anti-CD3 MAb must be present throughout the culture period for optimal proliferation and IL-2 responsiveness. The experiments depicted in Fig. 2 and Table VI examine the length of time T_4 cells require interaction with immobilized anti-CD3 to become IL-2 responsive and manifest maximal proliferation. In Table VI, T_4 cells were cultured in wells containing immobilized 64.1 for various time periods and then removed and cultured alone or with IL-2. Responses varied directly with the length of the initial culture. Moreover, in the absence of IL-2, responses were not observed unless the initial culture was longer than 14 h. The induction of responsiveness required somewhat more prolonged stimulation when immobilized OKT3 or immobilized OKT3 plus 60.3 was employed (Fig. 2). T_4 cells incubated with immobilized OKT3 or immobilized OKT3 plus 60.3 in the initial culture and then incubated alone did not proliferate unless the initial culture period exceeded 24 h. Proliferation noted in these cultures was never comparable to the response obtained when the preincubated cells were cultured with IL-2 or immobilized OKT3 with or without 60.3. Responses were detectable after only a 16-h preincubation with immobilized OKT3 with or without 60.3 when the second culture was supplemented with IL-2, but responses were always less than those noted when the second culture contained immobilized OKT3 with or without 60.3. T_4 cells proliferated vigorously when both cultures contained immobilized OKT3. The magnitude of the response, however, varied somewhat with the length of the initial culture. The responses of T_4 cells transferred after only a 6-h incubation were less than those transferred thereafter.

Discussion

These studies examined the effect of MAb directed at various T cell surface proteins on the capacity of immobilized anti-CD3 to induce T_4 cell proliferation in the absence of AC. Optimal proliferation induced by high density 64.1 was unaffected by the soluble or immobilized MAb used in these stud-

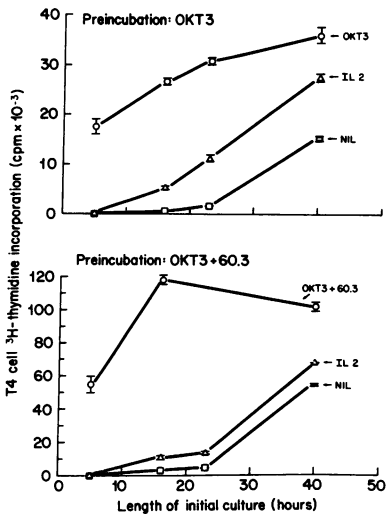

Figure 2. Requirement for continuous anti-CD3 stimulation for maximal T_4 cell proliferation. T_4 cells were cultured with immobilized OKT3 (200 ng/well) or OKT3 (200 ng/well) plus 60.3 (500 ng/well) for various time periods, harvested from the wells, and recultured alone, with IL-2 (10 U/ml) or the original stimulus. After a total of 3 d in culture, [^{3}H]thymidine incorporation was assessed and the data are expressed as the mean counts per minute of triplicate determinations±SEM.

Table VI. Prolonged Stimulation with Immobilized 64.1 Is Necessary for Optimal T_4 Cell Proliferation and IL-2 Responsiveness

Length of incubation with immobilized 64.1	T_4 cell DNA synthesis	
	Nil	IL-2
h	*[^{3}H]thymidine incorporation (cpm × 10^{-3})*	
4	0.1±0.1	7.5±0.8
14	1.6±0.1	29.9±2.0
19	3.9±0.1	37.0±6.3
23	5.8±0.1	73.1±4.5
38	67.9±1.3	98.0±4.4

T_4 cells (1×10^5) were cultured with immobilized 64.1 (200 ng/well) for various time periods, removed and cultured alone or with IL-2 (20 U/ml). After 3 d in culture, [^{3}H]thymidine incorporation was determined. The data are expressed as the mean counts per minute of triplicate determinations±SEM.

ies. In contrast, suboptimal stimulation of T_4 cells by low density 64.1 or immobilized OKT3 was markedly enhanced by immobilized but not soluble MAb directed at CD2, CD11a (LFA-1), or class I MHC encoded gene products. Responses were also increased by immobilized MAb to CD4 and both soluble and immobilized MAb directed at Tp44 (CD28) and CD5, although enhancement caused by these MAb was not as great as that elicited by immobilized MAb to CD2, LFA-1 or HLA-A,B,C. None of the immobilized or soluble MAb utilized in these studies, other than anti-CD3, had the capacity to induce T_4 cell proliferation alone or in the presence of soluble anti-CD3.

The capacity of MAb to T cell surface molecules to enhance immobilized anti-CD3 induced responses has been examined previously (27–30). In contrast to the current studies, however, the previous reports were only able to detect responses in cultures containing intact AC or supplemental IL-2. The current studies are the first in which T cell proliferation could be detected in the complete absence of AC or exogenous IL-2. These experiments, therefore, unambiguously delineate the signals that are necessary to induce T cell proliferation and not merely IL-2 responsiveness, since the cultures contain no other cell types. The capacity to induce proliferation and IL-2 responsiveness in the absence of AC relates to the use of MAb immobilized to polystyrene microtiter wells, which appear to have the capacity to bind a much greater density of MAb than more standard solid supports such as Sepharose or polystyrene beads. Previous studies have focused on the capacity of MAb to CD4, CD2, CD5, CD6, and CD8 to induce IL-2 responsiveness of anti-CD3 stimulated T cells (27–30). The current studies greatly expand upon this background and document that MAb to class I MHC molecules and LFA-1 are also able to enhance anti-CD3 induced T_4 cell proliferation. The costimulatory potency of anti-HLA-A,B,C and anti-LFA-1 was comparable to that of anti-CD2 and much greater than that of anti-CD4.

It is possible from the data obtained in the current and previous studies to create a hierarchy relating the capacity of the MAb to various T cell surface antigens to enhance immobilized anti-CD3 induced responses. Walker et al. demonstrated that the capacity of the MAb to enhance immobilized anti-CD3 induced responses depended on the molecule recognized by the MAb and not the epitope recognized by the MAb or the isotype of the MAb (30). Thus, responses were most effectively promoted by MAb to CD2 followed by MAb to CD5, CD6, and CD4 in descending order of potency. Whereas the capacity of MAb to CD6 to enhance immobilized anti-CD3 induced T cell proliferation was not examined in the current study, the enhancement induced by anti-CD2 was found to be greater than that induced by anti-CD4 as previously observed, despite the use of different MAb and different techniques of immobilizing the MAb. The current studies expand the previous observations by demonstrating that MAb to class I MHC molecules and LFA-1 are as effective as MAb to CD2 at enhancing anti-CD3 induced responses, whereas each is more effective than MAb to CD4, CD5, and CD28. This hierarchy has important implications. Anderson et al. (28, 29) and Emmrich et al. (28, 29) have used the capacity of immobilized MAb to CD4 to enhance anti-CD3 induced T cell IL-2 responsiveness to suggest that cross-linking of the T cell receptor/CD3 complex with CD4/8 is essential for T cell activation. If this reasoning is correct, the current findings and those of Walker et al. (30) would suggest that cross-linking CD3 with CD2 or LFA-1 may also be necessary and perhaps more important for T cell activation. Alternatively, the data suggest that cross-linking any one of a variety of T cell surface molecules to the T cell receptor/CD3 complex is sufficient to induce T cell activation when signaling via CD3 is suboptimal, although no such cross-linking is necessary when adequate triggering through CD3 occurs. Finally, the results with the MAb to class I MHC molecules indicate that costimulation of T_4 cells can also be achieved when separate signals are delivered. Thus, the finding that costimulation was noted when T_4 cells were cultured with MAb to CD3 and HLA-A,B,C immobilized to separate matrices indicates that in this circumstance T_4 cell activation is unlikely to require cross-linking of the T cell receptor/CD3 complexes to class I MHC molecules but rather results from the summation of separate signals generated when each of these molecules is cross-linked.

The mechanism whereby a second immobilized MAb enhances immobilized anti-CD3 induced T_4 cell activation has not been delineated. One possibility is that the additional immobilized MAb serves to bind the T_4 cell to the plastic microtiter well and thereby stabilizes the interaction between the CD3 molecule on the T_4 cell and the immobilized anti-CD3 MAb. This conclusion is supported by the observation that enhancement was most marked when T_4 cells were stimulated simultaneously with OKT3 and the additional immobilized MAb. This hypothesis is favored by Walker et al. (30). The authors support this claim with the observation that immobilized MAb to CD2, CD4, CD5, CD6, and CD8 did not enhance immobilized anti-CD3 induced IL-2 responsiveness when the MAb were immobilized to distinct surfaces. The finding that immobilized MAb to CD2 and CD4 do not enhance immobilized OKT3 induced responses when the MAb are immobilized onto different surfaces in the current studies confirms these findings. The current findings expand these observations by demonstrating that MAb to CD11a also do not enhance responses when immobilized onto a different surface. More importantly, however, the current findings demonstrate that immobilized MAb to class I MHC molecules do enhance immobilized OKT3 induced T_4 cell proliferation

when the MAb are immobilized onto different surfaces. These results demonstrate that MAb to class I MHC antigens enhance responses by a mechanism other than stabilizing the interaction between CD3 and anti-CD3 and support the conclusion that the interaction between class I MHC antigens and the immobilized MAb results in the generation of a signal that promotes T cell activation. These data indicate that MAb to class I MHC molecules differ from MAb to CD2, CD4, CD5, CD6, CD8, and CD11a in that they do not costimulate by cross-linking the molecule recognized with CD3, but rather deliver a separate and distinct signal to T_4 cells that can enhance activation and proliferation.

Several findings support the hypothesis that each of the immobilized MAb utilized augment anti-CD3 stimulated responses by delivering signals that promote T cell activation and not merely by binding the T cells to the microtiter wells and thereby facilitating interactions between CD3 and anti-CD3. First, in the studies by Walker et al. (30) and in the current studies, there was no correlation between the capacity of a MAb to augment anti-CD3 stimulated T cell proliferation or IL-2 responsiveness and the density of the recognized determinant, the epitope recognized by the MAb or the isotype of the MAb. Thus, in the current studies, immobilized anti-CD4 and anti-CD5 only enhanced responses modestly, although there is a high density of CD4 and CD5 molecules on the T cell surface (data not shown). Moreover, a 10-fold greater amount of anti-HLA-A,B,C compared to anti-CD2 was necessary to achieve comparable enhancement of immobilized OKT3 induced T_4 cell proliferation despite the observation that the density of class I MHC encoded gene products on T_4 cells is greater than the density of CD2 (data not shown). Finally, MAb to CD5 and CD28, that have the capacity to promote responses in the soluble form, promote responses less effectively than MAb to CD2, CD11a and HLA-A,B,C despite comparable expression of these determinants. These results all make it unlikely that passive promotion of CD3-anti-CD3 interactions by binding T_4 cells to the microtiter plates entirely explains the capacity of the second immobilized MAb to enhance anti-CD3 stimulated T_4 cell proliferation. It is more likely, therefore, that the additional immobilized MAb may promote anti-CD3 induced activation by directly signaling the T cell. The MAb to CD2, CD4, or LFA-1 may intercalate the recognized molecules between CD3 molecules and thereby enhance the strength of the stimulatory signal. This does not appear to be required for enhancement by MAb to class I MHC molecules. Thus, anti-CD3 may activate T_4 cells more effectively when CD3 is cross-linked to CD2, CD4, or LFA-1. By contrast, engagement and cross-linking of HLA-A,B,C appears to generate a unique signal that can costimulate T cells also activated by immobilized anti-CD3.

The capacity of MAb to CD2 to trigger T cell activation has been demonstrated previously (31–34). Therefore, the capacity of this MAb to enhance anti-CD3 induced responses is not surprising. The capacity of MAb to LFA-1 or HLA-A,B,C to enhance responses is, however, unexpected. Previously published results have suggested the possibility that class I MHC antigens might be involved in interactions with AC that support T cell activation. Thus, soluble MAb directed at class I MHC encoded gene products inhibited AC supported OKT3-induced proliferation (5), as well as responses induced by specific antigen and nonspecific mitogens (12, 35, 36), whereas antibodies to β_2 microglobulin have been shown to inhibit IL-2 production stimulated by allogeneic cells (37). Anti-HLA-A,B,C antibodies did not inhibit T cell proliferation induced by AC-independent stimuli such as the combination of OKT3 and phorbol myristate acetate (5). These results are consistent with the conclusion that AC-T cell interactions mediated by class I MHC antigens play a role in activating T cells. The current data indicate that these AC-T cell interactions can be simulated by immobilized MAb to HLA-A,B,C. Moreover, the findings suggest that cross-linking of class I MHC molecules delivers an independent signal that can enhance proliferation of T_4 cells stimulated by immobilized anti-CD3. More recent studies have supported this conclusion by demonstrating that MAb to class I MHC molecules stimulate a rise in intracellular free calcium in highly purified T_4 cells when cross-linked (Geppert, T. D., M. C. Wacholtz, L. S. Davis, and P. E. Lipsky, manuscript submitted for publication). These observations suggest that class I MHC molecules have the capacity to transmit activation signals to T_4 cells when cross-linked and support the conclusion that the enhancement of anti-CD3 induced proliferation by MAb to HLA-A,B,C results from the provision of an additional activation signal.

It is clear from previous studies that LFA-1 promotes cellular adhesion in a number of model systems (38, 39). T cell functions that are dependent on cellular interactions including antigen-, alloantigen-, and mitogen-induced T cell proliferation and cytotoxicity are inhibited by MAb to LFA-1 (7). The capacity of immobilized 60.3 to enhance immobilized OKT3-induced proliferation suggests that interactions involving LFA-1 may also deliver signals to the T_4 cell that promote anti-CD3-induced T_4 cell activation. Thus, one function of LFA-1, in addition to its role in cellular adhesion, may be to transmit regulatory signals. Previous work has demonstrated that a MAb to LFA-1 promotes murine B cell activation (40). In a similar manner, 60.3 may promote human T_4 cell activation by delivering a signal that facilitates proliferation induced by suboptimal stimulation via the CD3 pathway.

Previous studies have reported that CD4 plays a role in regulating T_4 cell function. Thus, anti-CD4 has been shown to inhibit antigen-, alloantigen-, and mitogen-induced T_4 cell proliferation (5, 8, 10). In contrast to MAb directed at CD2 and LFA-1, anti-CD4 MAb have also been demonstrated to inhibit accessory cell independent T_4 cell proliferation induced by immobilized anti-CD3 (6, 41). This finding has suggested that anti-CD4 may directly suppress T_4 cell activation. The findings presented here, that soluble anti-CD4 inhibits anti-CD3 stimulated T_4 cell proliferation, are in agreement with these earlier observations and demonstrate that interactions with CD4 can directly suppress T_4 cell responses. The capacity of anti-CD4 to inhibit T_4 cell responsiveness has been found to vary with the intensity of the activation signal (6). Thus, T_4 cell proliferation and IL-2 production induced by high density anti-CD3 were only modestly inhibited by soluble anti-CD4, whereas that stimulated by low density anti-CD3 was inhibited to a much greater degree. In contrast to the capacity of soluble anti-CD4 to inhibit, immobilized anti-CD4 enhanced immobilized OKT3-induced proliferation. These results are consistent with previous findings that cross-linking CD3 and CD4 can induce IL-2 responsiveness (28–30). Therefore, MAb directed at CD4 can transmit both negative and positive effects to T_4 cells. The current studies differ from previous reports (28–30) in that they demonstrate that immobilized anti-CD4 can facilitate proliferation of T_4 cells stimulated by immobi-

lized anti-CD3 even in the absence of exogenous IL-2. Thus, interactions involving CD4 molecules may enhance anti-CD3 induced T cell responses when the interacting ligand is bound to a matrix that effectively cross-links CD4.

Soluble MAb directed at CD5 or Tp44 (CD28) have been shown to enhance T_4 cell proliferation induced by anti-CD3 MAb (6, 9), and by fixed antigen bearing antigen presenting cells (42). The observation that 10.2 (anti-CD5) and 9.3 (anti-Tp44) were not more effective promoters of T_4 cell proliferation when immobilized rather than soluble suggests that these MAb do not simulate a signaling event that involves a physical interaction between T cells and accessory cells. Rather, the observation suggests that the putative physiologic ligands that interact with CD5 or Tp44 (CD28) may not be membrane bound and could be soluble growth factors. The subset of T_4 cells that responds to immobilized OKT3 in the presence of MAb to Tp44 (CD28) or CD5 appears to be smaller than that responding to anti-CD3 in the presence of immobilized MAb to CD2, LFA-1, or HLA-A,B,C. This supports the conclusion that circulating T_4 cells are not uniform in their activation requirements. This heterogeneity in signaling required for activation is most apparent when stimulation via the CD3 pathway is suboptimal.

In summary, the current studies demonstrate that anti-CD3 induced T cell activation is dependent on the epitope recognized by the anti-CD3 monoclonal antibody, the number of CD3 molecules recognized and the duration of the interaction with CD3. When the stimulus is suboptimal, T_4 cell activation is dependent on additional signals delivered by AC, and mimicked by monoclonal antibodies directed at CD28 (Tp44), CD5, CD4, CD2, LFA-1, or class I MHC molecules. Signals provided by these nonspecific interactions play a major role in determining the magnitude of T_4 cell responses.

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