Induction of Specific Nonresponsiveness in Unprimed Human T Cells by Anti-CD3 Antibody and Alloantigen

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Summary

Fresh peripheral blood mononuclear cells exposed to alloantigen for 3–8 d in the presence of anti-CD3 antibodies showed no response after restimulation with cells from the original donor but remained capable of responding to third-party donors. Antigen-specific nonresponsiveness was induced by both nonmitogenic and mitogenic anti-CD3 antibodies but not by antibodies against CD2, CD4, CD5, CD8, CD18, or CD28. Nonresponsiveness induced by anti-CD3 antibody in mixed leukocyte culture was sustained for at least 34 d from initiation of the culture and 26 d after removal of the antibody. Anti-CD3 antibody also induced antigen-specific nonresponsiveness in cytotoxic T cell generation assays. Anti-CD3 antibody did not induce nonresponsiveness in previously primed cells. Nonresponsiveness induced by anti-CD3 did not appear to be associated with suppressor cell activation. Thus, co-stimulation of the T cell receptor–CD3 complex on unprimed T cells with a fluid phase anti-CD3 antibody and allogeneic major histocompatibility complex antigens can induce either clonal anergy or clonal deletion. These results suggest novel approaches for achieving transplantation tolerance.

Antigen recognition by human T cells is mediated by the α and β polymorphic chains of the TCR, noncovalently associated on the cell surface with a group of five invariant polypeptides designated γ,δ,ϵ, and ζ-ζ, which collectively represent the CD3 complex responsible for signal transduction (1). Binding of an antibody to TCR or to CD3 can block T cell responses not merely by steric hindrance of TCR but also by altering cellular function (2, 3). The effects of TCR-CD3 binding by an antigen presented by an MHC gene product, a physiological solid-phase ligand, differ substantially from the effects of binding by a fluid phase ligand, such as a soluble antibody (4). An increase in the concentration of cytoplasmic free calcium and activation of protein kinase C can occur in either case, but IL-2 receptor expression is induced only with solid-phase ligands (4–6). Activation of the IL-2 gene requires TCR-CD3 binding by a solid-phase ligand together with certain other signals provided by accessory cells (7, 8). Soluble factors such as IL-1 and cell–cell interactions mediated through CD2, CD5, CD28, LFA-1, and MHC class I molecules can function as accessory activation signals (7–9). In the absence of such signals, it has been observed in type I murine T cell clones that solid-phase binding to TCR-CD3 induces anergy to further stimulation by specific antigen (reviewed in references 10, 11) and IL-2 (12).

After binding of fluid phase antibody, the TCR-CD3-ligand complex undergoes receptor-mediated endocytosis (4, 13, 14), but after binding of a solid-phase ligand internalization of the TCR-CD3 complex does not occur. The process of internalization, also referred to as modulation, is energy-dependent and appears to be regulated by the state of phosphorylation of the CD3γ subunit (15). After TCR-CD3 modulation, T cells become refractory to restimulation with specific antigen, PHA, Con A, or to signals delivered through CD2, CD5, and CD28 (4, 16–18). Reappearance of responsiveness, however, parallels reexpression of TCR-CD3 on the cell surface (16). In this report, we demonstrate that co-stimulation of unprimed human T cells with soluble anti-CD3 antibody and allogeneic MHC can induce a sustained state of antigen-specific nonresponsiveness.

Materials and Methods

Primary Mixed Leukocyte Culture. PBMC were prepared by density gradient centrifugation on Ficoll-Hypaque. PBMC were resuspended in medium containing RPMI 1640, 25 mM Hepes, 1 U/ml penicillin, 1 μg/ml streptomycin, and 10% pooled human serum that had been heat inactivated at 56°C for 30 min. Responder A and stimulators B and C were unrelated individuals chosen so that there was at least one HLA class I and one HLA-DR antigen mismatched between A and B, and A and C. Stimulator cell donors

B and C did not share HLA-DR, DQ, or DP antigens. 5×10^4 responder cells were mixed with 5×10^4 irradiated stimulator cells (3,000 rad) in round-bottomed 96-well plates. In certain experiments antibody containing medium was dispensed first, followed in sequence by responder and stimulator cells. Plates were cultured at 37°C in a 5% CO_2 atmosphere. Assays were performed in triplicate. Cultures were pulsed with one μCi of [^{3}H]thymidine 18 h before harvesting. 10 replicate plates were set up and one was harvested each day for 10 consecutive days. Data are reported as mean counts per minute of the three replicates.

Restimulation Assays. 10^7 PBMC from one individual were primed with an equivalent number of irradiated (3,000 rad) PBMC from another HLA class I and II incompatible individual in 25-cm^2 flasks, using identical medium and culture conditions as for primary MLC carried out in 96-well plates. Cells were cultured for 12 d before harvesting unless specified otherwise. For blocking experiments, cells were cultured for 8 d in the presence of antibody, washed three times, recultured in medium without antibody for four additional days, harvested on day 12, and then restimulated. In experiments of tertiary stimulation, a secondary culture was carried out in flask, as in the first. For assay, 2×10^4 primed responders and 5×10^4 irradiated stimulators were incubated in 96-well round-bottomed wells in medium without antibody. Assays were performed as detailed for primary MLC. Percent control response of antibody-treated cells was calculated by the formula: $100 \times$ [cpm (α_E) − cpm (β_E)]/[cpm (α_C) − cpm (β_C)], where: α is allogeneic MLR, β is autologous MLR, E is experimental antibody, and C is control antibody.

Suppression Assays. Lymphocytes were primed as described for use in restimulation assays. On day 12, primed cells were irradiated with 1,500 rad and tested as regulators in a primary MLC. 10^4 regulators were mixed with 5×10^4 fresh autologous responder PBMC and 5×10^4 irradiated (3,000 rad) stimulators per well. Proliferation was measured daily for 10 d. The response to irradiated autologous stimulators was consistently <2% of the response of allogeneic stimulators and was not affected by primed regulators. Percent inhibition was calculated by the formula: $100 \times \{1-$[cpm (responder + stimulator$_x$ + regulator$_x$)]/[cpm (responder + stimulator$_x$)]$\}$.

Generation of CTL. Freshly separated PBMC or primed lymphocytes were tested for CTL precursor activity by priming in a modified MLC. Responder cells (10^7) either fresh or primed as specified for each experiment, and irradiated stimulator cells (10^7), were cultured for 6 d, harvested, washed twice, and tested for cytolytic effector activity in a 4-h ^{51}Cr-release assay against PHA blasts. Both autologous or stimulator lymphocytes were tested as target cells. Maximum and spontaneous release values were obtained by incubating targets with 1% Triton-X 100 and medium alone, respectively. Triplicate assays were carried out at E/T ratio of 10:1, 50:1, 100:1 in V-bottomed 96-well plates. Data are reported as mean percent specific ^{51}Cr release.

Antibodies. Murine mAbs used in this study are listed in Table 1. Antibodies WT31 (anti-CD3-α/β complex), X39 and X40 (anti-KLH), and 2A3 (anti-CD25) were obtained from Becton Dickinson (San Jose, CA). Antibodies 1A12 and T11D7 (Anti-murine Thy-1.1) were kindly provided by Dr. Irv Bernstein (Fred Hutchinson Cancer Research Center, Seattle, WA). All other antibodies were produced in our laboratory and were previously described (see references 19–28 cited in Table 1) with the exception of two of them. Antibodies BC3 and BC18 are products of hybridomas generated by fusion of the HGPRT$^-$ myeloma cell line BALB/c MOPC21 NS1/1 provided by Dr. Caesar Milstein (Molecular Research Council, Cambridge, UK) with splenocytes obtained from BALB/c mice after multiple intraperitoneal immunizations with 2×10^6 PHA-stimulated mononuclear cells from normal human volunteers. The specificity of antibody BC3 for CD3 was determined by immunoprecipitating two distinct species of 19,000 and 29,000 M_r from ^{125}I-labeled PBL and by comodulation experiments with anti-CD3 antibody 64.1 (20). Antibody BC3 specificity for the CD3ϵ subunit was documented by staining of the $\epsilon\Delta$C2 cell line (kindly provided by Dr. Ellis Reinherz, Dana Farber Cancer Institute, Boston, MA), which was generated by transfecting the human CD3ϵ cDNA into the 3DO54.8 murine T cell line (29). In contrast to antibody BC3, the anti-CD3 antibody 38.1 did not bind $\epsilon\Delta$C2, suggesting that it recognizes another CD3 subunit or an epitope on the ϵ chain not expressed by the transfectant. The anti-CD3 antibody 64.1 demonstrated very weak binding to $\epsilon\Delta$C2. Antibody BC3 blocked binding of FITC-conjugated antibody 64.1 or 38.1 to human T cells, but neither 64.1 nor 38.1 blocked binding of FITC-conjugated BC3. Finally, antibody 64.1 blocked binding of FITC-conjugated 38.1 to human T cells, but the reverse did not occur. Taken together, these data suggest that BC3, 64.1, and 38.1 bind to closely associated but distinct epitopes expressed on the CD3 complex. The specificity of antibody BC18 for CD2 was determined by immunoprecipitating a 50,000 M_r species from ^{125}I-labeled PBMC and by blocking lymphocyte adhesion to sheep erythrocytes. Before use in functional studies, mAbs were purified from ascites fluids by precipitation with 50% saturated ammonium sulfate followed by DEAE-Sephacryl chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) or by affinity chromatography on Sepharose-bound protein A. Purified antibodies were dialyzed against PBS and filter sterilized.

Immunofluorescence Analyses. Surface expression of the CD3 complex or the CD3-associated TCR-α/β complex was quanti-

Table 1. *mAbs Used in this Study*

Clone designation	Ig subclass	Antigen recognized	Reference
9.6	IgG2a	CD2	19
BC18	IgG1	CD2	This paper
38.1	IgM	CD3	20
64.1	IgG2a	CD3-ϵ	20
BC3	IgG2b	CD3-ϵ	This paper
WT31	IgG1	CD3-α/β complex	21
66.1	IgM	CD4	20
10.2	IgG2a	CD5	22
51.1	IgG2a	CD8	23
60.3	IgG2a	CD18	24
2A3	IgG1	CD25	25
9.3	IgG2a	CD28	26
X40	IgG1	KLH	*
X39	IgG2a	KLH	*
9E8	IgG2a	p15(E)	27
1A12	IgG2b	Thy1	28
T11D7	IgM	Thy1	28

* See Materials and Methods.

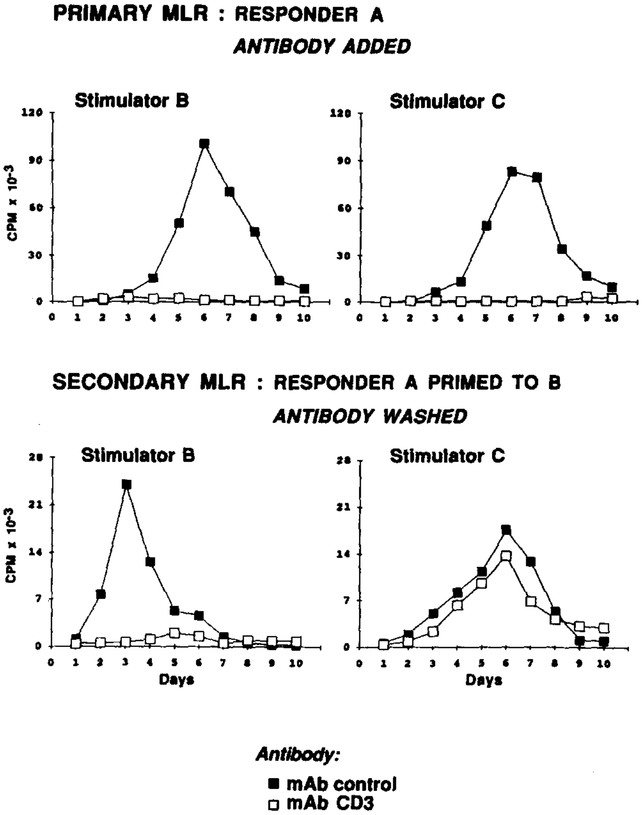

Figure 1. Induction of antigen specific nonresponsiveness by anti-CD3. For the primary MLR, 50,000 responder cells from donor A were stimulated with 50,000 irradiated stimulator cells from donor B (*top left panel*) and donor C (*top right panel*) in the presence of anti-CD3 antibody BC3 (*open square*) or a nonbinding control antibody (*closed square*). The cpm of cultured cells incubated with autologous stimulator cells with BC3 or control antibody were not different from each other and both were <2% of the response to allogeneic stimulators in the presence of control antibody (not shown). For the secondary MLR, responders from donor A primed with cells from donor B in the presence of BC3 (*open square*) or control antibody (*closed square*) were restimulated with cells from donor B (*left bottom panel*) and C (*right bottom panel*) in the absence of antibody.

tated by microfluorimetry of cells stained respectively with FITC-conjugated antibody BC3 (conjugated with the method of Goding [30]) or with FITC-conjugated antibody WT31 (purchased from Becton Dickinson). Controls were provided by cells stained with isotype-matched antibodies of irrelevant specificity. Cells (5 × 10^5) were incubated at 4°C for 30 min in 25 μl of medium containing 0.1% sodium azide and a saturating concentration of antibody. Cells were then washed, fixed in 1% paraformaldehyde, and analyzed on a FACScan using C30 software (Becton Dickinson).

Results

Induction of Antigen-specific Nonresponsiveness by Anti-CD3. Fresh human PBMC were incubated for 1 h at 37°C with 10 μg/ml of either the anti-CD3 murine IgG2b mAb BC3 or a nonbinding antibody of irrelevant specificity. The antibody-treated PBMC were then mixed with irradiated, HLA-incompatible stimulator cells and cultured in the continuous presence of antibody. Antibody BC3 did not induce proliferation of cells cultured with irradiated autologous stimulators (data not shown) and inhibited completely the proliferative response of cells stimulated with allogeneic stimulators from two different donors (Fig. 1, *top panels*). To evaluate effect of antibody BC3 on secondary responses, lymphocytes were cultured with alloantigen for 8 d in separate flasks in medium containing BC3 or control antibody. Cells were then washed to remove antibody, cultured in fresh medium for an additional 4 d to allow reexpression of the CD3-TCR complex (as demonstrated in Fig. 2), and then restimulated with irradiated PBMC from either the original donor (Fig. 1, *left bottom panel*) or from a third-party donor (Fig. 1, *right bottom panel*) in medium containing no antibody. Cells primed in the presence of control antibody (*closed symbols*) and restimulated with PBMC from the donors originally used for priming showed a typical accelerated secondary proliferative response, peaking on day 3. In contrast, those same primed cells showed a typical primary response, peaking on day 6, when stimulated with PBMC from a third-party donor. Cells primed

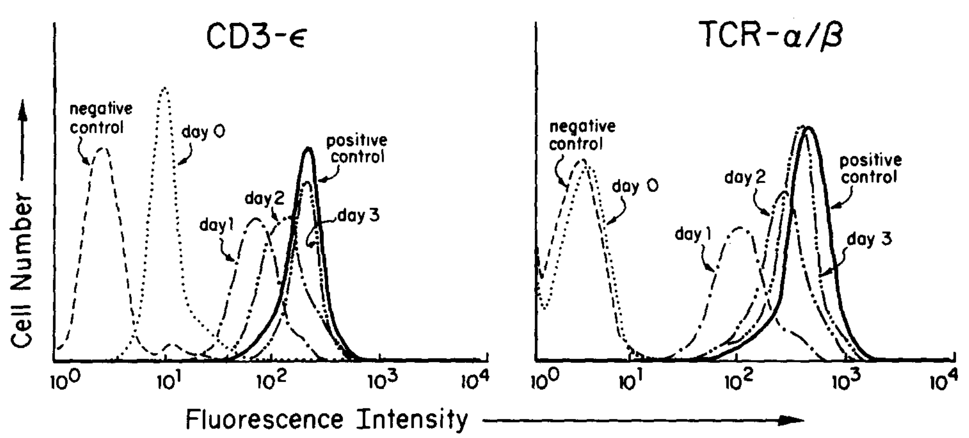

Figure 2. Reexpression of CD3 and TCR-α/β molecules after modulation induced by anti-CD3. Cells were cultured in primary MLC for 8 d in the presence of 10 μg/ml of anti-CD3 antibody BC3, washed and recultured in medium containing no antibody. Cells were stained by direct immunofluorescence immediately after washing, time defined day 0 (. . .), on day 1 (_ . _), day 2 (_ . . . _), or day 3 (_ . . . . _) with FITC-conjugated antibody BC3 (*left*) or CD3-α/β-specific antibody WT31 (*right*). Negative controls (_ _) were stained by a nonbinding, isotype-matched FITC-conjugated antibody of irrelevant specificity. Positive controls (_) were cells primed in the presence of a nonbinding antibody.

in the presence of antibody BC3 (*open symbols*), however, showed no detectable response when challenged with PBMC from the original donor, yet responded normally to PBMC from a third-party donor. The degree of proliferation observed in secondary MLC correlated with the concentration of antibody BC3 in the primary cultures, with 93% inhibition achieved at 10 μg/ml, and 73% inhibition at 1 μg/ml (data not shown). These results demonstrate that the secondary proliferative response of human T cells can be inhibited in an antigen-specific manner when the primary culture with alloantigen occurs in the presence of antibody BC3 at a concentration of 10 μg/ml.

To assess whether the ability to induce alloantigen-specific nonresponsiveness was unique for antibody BC3, other anti-CD3 antibodies were tested in identical assays. Antibody 38.1 is a murine IgM anti-CD3 antibody that is not mitogenic for human PBMC unless bound to a solid-phase matrix. Antibody 38.1 inhibited primary MLR by 85% when compared with a control antibody of irrelevant specificity (Table 2, line 2). Furthermore, cells exposed to alloantigen in primary MLC in the presence of antibody 38.1 demonstrated a decreased response to the specific antigen in restimulation assay, as low as 15% of control values, whereas response to a third-party alloantigen was not affected. Antibody 64.1 is a murine IgG2a anti-CD3 antibody that is mitogenic for human PBMC presumably by virtue of crosslinking the CD3 complex on T cells with the Fc receptor type I (CD64) on monocytes (31, 32). The degree of proliferation of PBMC incubated with antibody 64.1 was similar in response to either autologous or allogeneic irradiated stimulators, so that the response to the allogeneic stimulus was as low as 3% of control cultures with no antibody (Table 2, line 3). Cells exposed to alloantigen in the presence of antibody 64.1 had a decreased response to the specific antigen in restimulation assay, as low as 4% of control values, whereas response to third-party alloantigen was 45% of control values. Therefore, T cell nonresponsiveness to specific alloantigen was induced by three distinct anti-CD3 antibodies regardless of their mitogenic effect on T cells.

Table 2. *Effect of Anti-Lymphocyte Antibodies in Primary MLC on T Cell Responses to Primary and Secondary Stimulation by Alloantigen*

Antibody added to primary MLC		Percent peak proliferative control response		
			Secondary MLC	
Specificity	Clone	Primary MLC	Specific antigen	Third-Party antigen
CD3	BC3	<u>1</u>	<u>7</u>	79
CD3	38.1	<u>15</u>	<u>15</u>	135
CD3	64.1	<u>3</u>	<u>4</u>	45
CD18	60.3	<u>1</u>	71	130
CD2	BC18	<u>10</u>	108	81
CD2	9.6	<u>13</u>	72	81
CD28	9.3	34	109	118
CD4	66.1	56	93	100
CD5	10.2	83	54	103
CD8	51.1	92	60	122
Control	9E8	100	100	100

Primary cultures were performed in medium containing 10 μg/ml test or control antibody. Secondary cultures were performed in medium containing no antibody. [^{3}H]TdR uptake was measured daily for 10 d and cpm values at peak response were used to calculate percent of the control proliferative response as described in Materials and Methods. The timing of peak proliferation differed between primary and secondary MLC but was identical for each antibody tested. The distribution of the values shown in the table is bimodal and values clustering around the lower mode are reported in underscored characters.

Secondary Proliferative Response of Lymphocytes Exposed to Alloantigen in the Presence of Antibodies to Other Cell Surface Molecules. Certain antibodies specific for lymphocyte surface receptors other than CD3 have profound effects on T cell function. Antibodies against CD18 (the β chain of the leukocyte function–associated antigen 1 [LFA-1 complex]) and CD2 (LFA-2) inhibited lymphocyte proliferation in primary MLC (Table 2, lines 4, 5, and 6) but had no effect on kinetics or magnitude of subsequent secondary proliferative responses to specific or third-party alloantigens in absence of antibody. Anti-CD28 antibody 9.3 and anti-CD4 antibody 66.1 inhibited primary MLR by 66% and 44%, respectively, but had no effect on secondary proliferative responses in the absence of antibody (Table 2, lines 7 and 8). Anti-CD5 antibody 10.2 and anti-CD8 antibody 51.1 did not inhibit primary or secondary MLR (Table 2, lines 9 and 10). Taken together, these data indicate that anti-CD3 antibodies are unique in their ability to induce nonresponsiveness to restimulation with specific alloantigen in secondary cultures.

Duration of Antigen-specific Nonresponsiveness Induced by Anti-CD3. To assess the duration of alloantigen-specific nonresponsiveness, cells were exposed to alloantigen in medium containing BC3 or control antibody, washed on day 8, and cultured in fresh medium without antibody or alloantigen for 4, 11, or 18 d. These cells were restimulated on days 12, 19, and 26, respectively, after initiation of the primary culture. At all time points, cells primed in medium containing control antibody showed a typical accelerated proliferative response, peaking on day 3, when restimulated with irradiated PBMC from the donor originally used for priming and showed a typical primary response, peaking on day 6, when restimulated with irradiated PBMC from a third-party donor (Fig. 3). In contrast, cells primed in the presence of anti-CD3 antibody did not respond when stimulated with irradiated PBMC from the original donor but remained capable of responding to cells of a third-party donor. These data demonstrate that the nonresponsive state induced by exposure to alloantigen in the presence of antibody BC3 appears to last for at least 34 d after initiation of the culture and for at least 26 days after removal of the antibody.

Time Required for Anti-CD3 to Induce Nonresponsiveness. To

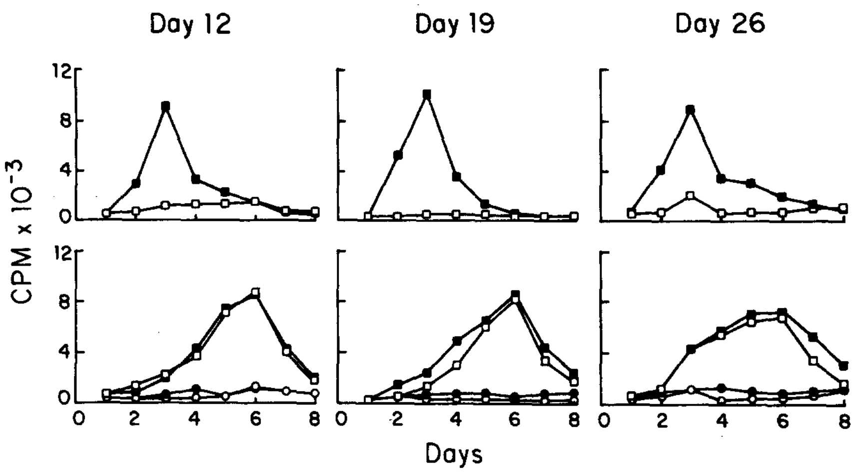

Figure 3. Duration of antigen-specific nonresponsiveness induced by anti-CD3. Responder cells primer with allogeneic stimulators in the presence of anti-CD3 (*open symbols*) or control antibody (*closed symbols*) were restimulated at the indicated time points with cells from the same donor (*top*) or a third-party donor (*bottom, squares*), or with autologous cells (*bottom, circles*) in the absence of antibody.

determine the duration of exposure to anti-CD3 necessary for development of nonresponsiveness, cells were washed on days 1, 3, 5, or 7 of primary MLC, resuspended in fresh medium without antibody, and rested until day 12 when they were restimulated with irradiated PBMC from the original donor or from a third-party donor. Primary MLC in the presence of antibody BC3 for 3, 5, or 7, but not for 1 d, produced nonresponsiveness in secondary cultures stimulated with irradiated PBMC from the original donor and had no effect on the response to irradiated PBMC from third-party donors. Primary MLC carried out with antibody BC3 for 1 d had diminished the magnitude of the secondary response to 68% of the control (Fig. 4). Thus, it appears that the minimum time of exposure to antibody BC3 needed to induce alloantigen-specific nonresponsiveness in MLC is between 1 and 3 d.

Effect of Anti-CD3 on CTL Generation. Disparity for MHC class II antigens induces proliferation of allogeneic T cells in MLC, while disparity for MHC class I antigens induces the generation of cytotoxic T cells. To determine whether antibody BC3 added to the primary MLC could block the generation of CTL as well as cell proliferation, cultures were set up in medium containing BC3 or control antibody for 5 d. These cells were washed, cultured in fresh medium without antibody for 3 d, and then tested in a standard 4-h ^{51}Cr-release cytotoxicity assay against target cells from the specific donor. Antibody BC3 abrogated generation of CTL (data not shown), a finding previously demonstrated for other anti-CD3 antibodies (2). In subsequent experiments, cultures containing BC3 or control antibody were maintained for 8 d, cells were washed and cultured in fresh medium without antibody for 4 d. Cells were then restimulated with irradiated PBMC from the original donor or from a third-party donor and tested for cytolytic activity. Cells primed in the presence of control antibody (*closed symbols*) were able to generate cytotoxic activity when restimulated with cells of both the original donor (Fig. 5, *left*) and the third-party donor

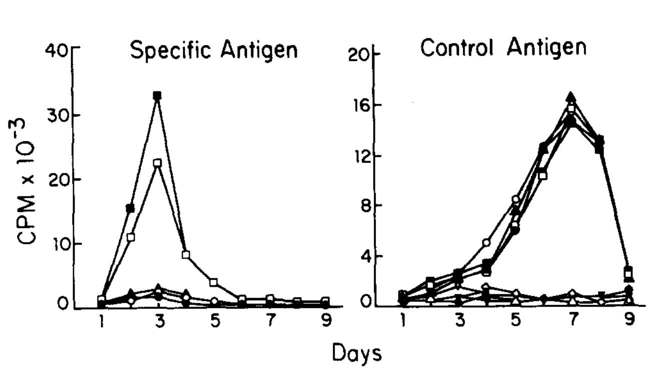

Figure 4. Time required for anti-CD3 to induce nonresponsiveness. Responder cells primed with allogeneic stimulators in the presence of control antibody (■) or anti-CD3 for 1 (□), 3 (●), 5 (O), or 7 (▲) d were restimulated with cells from the same donor (*left*) or a third-party donor (*right, upper traces*) or autologous cells (*right, lower traces*: control antibody [◆], anti-CD3 for one [◇], 3 [▼], 5 [▽], or 7 [△] d) in the absence of antibody.

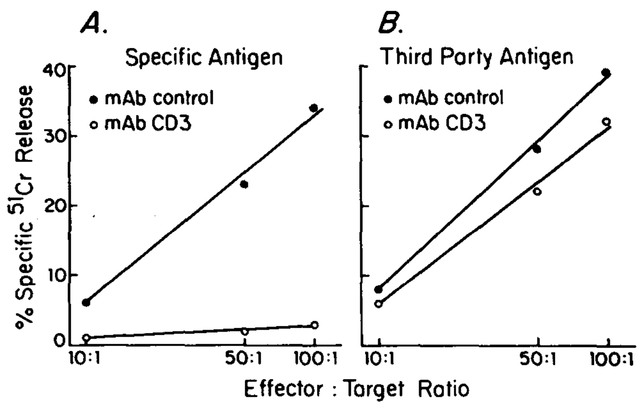

Figure 5. Effect of anti-CD3 on CTL generation. Responder cells were primed in the presence of anti-CD3 (*open circle*) or nonbinding control antibody (*closed circle*) and then restimulated with cells from the same allogeneic donor (*left*) or from a third party donor (*right*) in the absence of antibody. Cytotoxic activity against T lymphoblasts from the respective donors was assayed on day 6 of the secondary cultures.

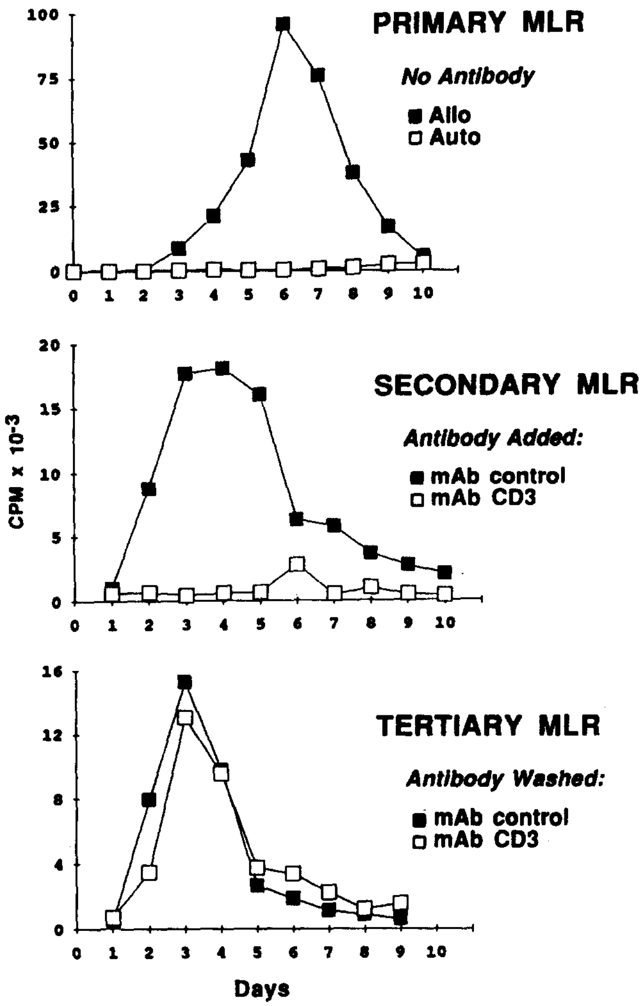

Figure 6. Effect of anti-CD3 on responsiveness of primed cells. PBMC were primed with allogeneic stimulators in absence of antibody (*top*). Primed cells were restimulated in secondary MLR with cells from the same donor (*middle*) in the presence of anti-CD3 (*open square*) or control antibody (*closed square*). Cells from respective bulk cultures were restimulated in a tertiary MLR (*bottom*) with cells from the same donor in absence of the antibody.

(Fig. 5, *right*). In contrast, cells primed in the presence of antibody BC3 (*open symbols*) did not generate cytotoxic activity when restimulated with cells from the original donor but remained capable of generating cytotoxic activity when restimulated with cells from the third-party donor. Thus, findings in the cytotoxicity assay paralleled those seen in the proliferative assays.

Effect of Anti-CD3 on Responsiveness of Primed Cells. Further experiments were undertaken to determine whether alloantigen-specific nonresponsiveness could be induced in primed cells. For these experiments, cells were primed in a 12-d MLC in medium containing no antibody (Fig. 6, *top panel*). Primed cells were then incubated with BC3 or control antibody for 1 h at 37°C, and restimulated with cells from the donor originally used for priming. Antibody BC3 completely inhibited the secondary proliferative response (Fig. 6, *middle panel*). In separate cultures, primed cells were restimulated in the presence of BC3 or control antibody for 8 d, then washed and rested for four additional days. Cells were then assayed in a tertiary MLR. Antibody BC3 had had no effect on responsiveness of primed cells to restimulation with specific antigen (Fig. 6, *bottom panel*). Thus, induction of nonresponsiveness by antibody BC3 was seen only in unprimed and not in primed cells.

Effect of Anti-CD3 on Generation of Cells with Suppressive Activity. During an in vitro MLC, certain cells acquire antigen-specific suppressive activity (33). This can be demonstrated by a reduced proliferation when primed irradiated cells are added to fresh autologous responders stimulated with specific alloantigen. Suppressive activity is increased if the priming MLC is carried out in the presence of cyclosporine or antibodies binding to the IL-2 receptor (34, 35). We investigated whether cells primed to specific antigen in the presence of antibody BC3 had acquired this type of suppressive activity. Cells recovered from primary MLC carried out in the presence of antibody BC3 were irradiated and added as regulators in a fresh MLC. Cells primed in the presence of the anti IL-2 receptor light chain antibody 2A3 (CD25) at 10 μg/ml or cyclosporine A at 0.5 μg/ml suppressed the

Table 3. *Effect of Anti-CD3 mAb on Generation of Cells with Suppressive Activity*

Cell donor		Percent inhibition			
Priming	Stimulating	Antibody 9E8 (negative control)	Antibody BC3 (anti-CD3)	Antibody 2A3 (anti-CD25)	CYA
A	A	32	34	87	93
A	B	9	4	7	11
B	B	32	20	85	88
B	A	15	10	−10	0

Primary cultures were performed in medium containing 10 μg/ml 9E8 (control), BC3 (anti-CD3), 2A3 (anti-CD25), or 0.5 μg/ml cyclosporine A (CYA), mixing responder cells from one individual with irradiated stimulator cells from HLA incompatible donors A or B. Primed cells were irradiated (1,500 rad) and tested as regulators in a fresh MLC. [^{3}H]thymidine uptake was measured daily for 10 d and cpm values at peak response were used to calculate percent inhibition as described in Materials and Methods. No significant differences in response kinetics were observed for antibody or CYA treated cultures (data not shown).

proliferative response of fresh autologous lymphocytes stimulated with specific alloantigen but had no effect on the response to alloantigen from third party donors (Table 3). In contrast, cells primed in control cultures with an antibody of irrelevant specificity had little specific suppressive activity. Cells primed in the presence of anti-CD3 also showed little specific suppressive activity. Thus, nonresponsiveness induced by anti-CD3 antibody was not likely the result of activation of a radiation-resistant suppressor cell.

Discussion

Our study demonstrates that co-stimulation with a soluble anti-CD3 antibody and alloantigen can induce a sustained state of specific nonresponsiveness in unprimed human T cells. One possible interpretation of this phenomenon is that modulation of the TCR-CD3 complex by a high concentration of soluble anti-CD3 antibody can condition T cells to become anergic in response to allogeneic MHC, a process that might be similar to induction of anergy by excess antigen (36, 37). We demonstrated nonresponsiveness in both proliferative as well as CTL generation assays, suggesting that anti-CD3 antibodies can condition the response of more than one type of T cell to both MHC class I and class II alloantigens. Lack of CTL generation, however, might merely reflect the absence of helper activity and does not necessarily indicate nonresponsiveness to MHC class I antigens.

Nonresponsiveness was inducible only by antibodies specific for CD3 and not by antibodies to other T cell surface molecules. LFA-1 (CD11/CD18) and LFA-2 (CD2) are surface receptors mediating antigen-independent intercellular adhesion by binding to their respective ligands ICAM-1/2 and LFA-3 expressed on a variety of all types. LFA-1 and LFA-2 or associated structures, are capable of signal transduction since antibodies to LFA-1 and LFA-2 not only can block cell adhesion but also effect a variety of other cellular functions. We have confirmed that anti-LFA-1 and anti-LFA-2 antibodies inhibit primary MLR, but this does not affect the pattern of secondary response to specific antigen. Results of anti-LFA-1 and LFA-2 blocking experiments suggested that T cell memory can be induced with little or no DNA synthesis. Thus, it would seem unlikely that nonresponsiveness induced by anti-CD3 antibodies results solely from insufficient cellular proliferation during primary antigen exposure.

The culture conditions we used were favorable for allowing sustained T cell viability and function as measured by responses to alloantigens for up to 26 d after initiation of the primary culture. Nonresponsiveness to restimulation by the priming antigen did not likely result from failure to reexpress the TCR-CD3 complex, since cells showed a uniform pattern of TCR and CD3 surface reexpression, that had returned to control levels within 3 d of culture in absence of anti-CD3 antibody. Lack of TCR-CD3 reexpression on the small population of clones specific for the priming antigens, however, could not be excluded by our assay. We also attempted to rule out that nonresponsiveness to the priming antigen was secondary to activation of suppressor cells, which occurs in MLC. Cells able to suppress the proliferative response of unprimed autologous PBMC against specific alloantigen can be preferentially activated in MLC carried out in the presence of cyclosporine A or antibodies to the IL-2R 55-kD chain (34, 35). Cells exposed to anti-CD3 and alloantigen were nonresponsive to restimulation in secondary MLC, but had no detectable suppressive effect when added to autologous responder PBMC in a fresh MLC against specific alloantigen. By a process of elimination, we suggest that T cell nonresponsiveness induced by anti-CD3 antibody is likely the result of clonal anergy or death. The MLC model cannot distinguish these two possibilities.

Use of nonmitogenic murine anti-CD3 antibodies was essential to achieve the results shown. Mitogenic anti-CD3 antibodies also induced nonresponsiveness, although culture with mitogenic anti-CD3 yielded lower cell viability, which might in part account for the decreased secondary response against cells from third-party donors. In addition, mitogenic anti-CD3 antibodies by themselves might induce generalized T cell hyporesponsiveness, as demonstrated by in vitro and in vivo experiments in murine systems (11, 38). The mitogenic activity of anti-CD3 antibodies depends on their heavy chain isotype (39). Since Fc receptors on accessory cells provide a matrix for multimeric binding of anti-CD3 antibodies, and since crosslinking of CD3 molecules is one of the requirements for induction of DNA synthesis in T cells, the affinity of Fc receptors on accessory cells for the Fc domain of anti-CD3 antibodies represents an important determinant of T cell activation. Fc receptors types I (CD64) are permissive for the mitogenic activity of murine IgG2a and IgG3 anti-CD3 antibody (31), while Fc receptors type II (CD32) are permissive for murine IgG1 anti-CD3 antibodies (40). Fc receptors type II on monocytes and B cells bind aggregated murine IgG2b immunoglobulins, but this does not lead to mitogenic activity, even though anti-CD3 IgG2b antibodies are mitogenic when bound to a solid-phase support (40). This may reflect the high flexibility of the hinge region of murine IgG2b immunoglobulins that allows the bound antigen to move in multiple directions (41).

The functional consequences of ligand binding to TCR-CD3 depend both on the physical properties of the interaction and the state of differentiation and activation of the T cell. In immature thymocytes and in certain T cell tumor lines, crosslinking TCR-CD3 induces an increase in cytoplasmic free calcium followed by RNA and protein synthesis (42). Cells increase in size but are blocked in progression through the cell cycle at the interface between G1 and S (43). Activation of a calcium dependent endonuclease causes DNA fragmentation and cell death, a phenomenon known as apoptosis (44–51). It has been suggested that clonal deletion of autoreactive T cells in the thymus may occur by a similar process (52). Variants of a murine T cell hybridoma expressing normal amounts of CD3ζ_2 but decreased amounts of CD3$\zeta\eta$ demonstrated little activation of a serine-specific protein kinase that phosphorylates CD3γ (45). In contrast to the parental line, the variants did not undergo programmed cell death after CD3 crosslinking (50). In this and in another model (53), the biological responses of T cells seemed to depend on the composition of the TCR-CD3 complex. In view of this observation, it is attractive to question whether the TCR-CD3 complex on human T cells undergoes structural changes after a soluble anti-CD3 has induced internalization of the TCR-CD3-ligand complex.

In contrast to lymphocytes freshly separated from peripheral blood, we could not induce nonresponsiveness in T cells previously primed in vitro. Profound differences have been identified between "naive" and "memory" T cells (54). Memory T cells can be distinguished by their increased expression of LFA-1, LFA-2, LFA-3, ICAM-1, CD29, and CD45RO, whereas naive T cells have low or undetectable expression of these molecules, and instead, express high levels of CD45RA. During in vitro priming, T cells stimulated by alloantigen lose the cell surface phenotype of naive T cells and acquire a phenotype characteristic of memory T cells and requirements for further cellular activation change (55). Proliferative responses to CD3 crosslinking are greater for memory T cells than for naive T cells, possibly as a result of more robust accessory signals delivered through newly expressed adhesion receptors. The question relevant to our model is whether CD3 modulation induced by fluid phase anti-CD3 antibody inhibits expression of activation antigens such as LFA-1, LFA-2, LFA-3, ICAM-1, ICAM-2, and CD28 induced by solid-phase TCR-CD3 ligands (such as allogeneic MHC). If this were the case, T cells bound by soluble anti-CD3 would lack adhesion structures for accessory cells. Thus, there would be a similarity between nonresponsiveness induced by anti-CD3 antibody and the anergy occurring in type 1 murine T cell clones after solid-phase ligand binding to TCR-CD3 in absence of accessory signals (10). Alternatively, the ability to induce nonresponsiveness in naive T cells but not in memory T cells may reflect differences in the structure of the TCR-CD3 complex or in the types of signals transduced.

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