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Immunology

Identification of a monoclonal antibody specific for a murine T3 polypeptide

(T-cell antigen receptor/mouse/T-cell activation)

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ABSTRACT A monoclonal antibody (145-2C11) specific for the murine T3 complex was derived by immunizing Armenian hamsters with a murine cytolytic T-cell clone. The antibody is specific for a 25-kDa protein component (T3-ε) of the antigen-specific T-cell receptor. It reacts with all mature T cells and can both activate and inhibit T-cell function. These results identify T3-ε as a cell surface protein involved in the transduction of activation signals.

Activation of mature T cells is a process that requires both receptor occupancy by foreign antigen in the context of major histocompatibility complex (MHC) proteins and transduction of a transmembrane signal. The specific antigen recognition has been shown to be dependent on a disulfide-linked heterodimer containing two integral membrane glycoprotein chains, α (45–50 kDa) and β (40–44 kDa) (1). The α and β chains are encoded by families of genes, giving rise to a polymorphic set of cell membrane receptors clonally expressed on T lymphocytes. Dembic *et al.* (2) and Saito *et al.* (34) have demonstrated that transfection and expression of the genes encoding the $\alpha\beta$ heterodimer results in the acquisition of MHC-restricted antigen recognition, establishing the direct relationship of antigen specificity to $\alpha\beta$ expression. The second step of T-cell activation (transmembrane signaling) is presently thought to be mediated by an invariant complex of proteins (T3) that by immunoprecipitation has been shown to be noncovalently associated with the $\alpha\beta$ heterodimer in the T-cell receptor (TCR) complex (3, 4). The human T3 complex consists of at least three polypeptides, two glycosylated proteins of 25 kDa and 20 kDa (T3-δ and T3-γ, respectively) and an endoglycosidase F-insensitive protein of 20 kDa (T3-ε) (5). The murine T3 structure has been identified as a similar complex of low molecular mass proteins, including T3-δ, T3-ε, T3-γ, and a 16-kDa polypeptide present as a 32-kDa homodimer (T3-ζ) (6–8). The role of the T3 proteins in transmembrane signaling is substantiated by the finding that several of the T3 components have a long intracellular polypeptide portion (9–11) that is phosphorylated upon $\alpha\beta$ heterodimer occupancy (12). In addition, monoclonal antibodies (mAbs) binding to the human T3-δ molecule can result in a rise in cytoplasmic free calcium (13), metabolism of phosphatidylinositol phosphates (14), T-cell proliferation (15), lymphokine production (16), and cytolysis (17, 18). Recently, mAbs to T3 have allowed the identification of a subpopulation of human T cells that express T3 but not the $\alpha\beta$ heterodimer (19, 20). In these cells, the T3 components are associated on the cell surface with polypeptides other than α and β, including one that appears to be the product of the γ gene (19). Anti-T3 mAbs have been shown to activate this T-cell subset, indicating that T3 might also be involved in the signaling of activation signals initiated by the recently described TCR_{γ} receptor complex (20).

Unlike the study of human systems, the study of murine T-cell activation and ontogeny has been hampered by the lack of monoclonal antibodies able to detect cell surface expression of murine T3-associated structures. In this paper we describe a mAb specific for the murine T3 complex. The antibody was generated by immunizing Armenian hamsters with a murine T-cell clone and was identified by means of a screening assay designed to detect mAbs directed at cell surface molecules involved in murine T-cell activation. This mAb reacts with a 25-kDa protein (identified as T3-ε) that is noncovalently associated on the cell surface with the antigen-specific $\alpha\beta$ heterodimer and has functional properties similar to anti-human T3-δ antibodies.

MATERIALS AND METHODS

Animals and Cell Lines. Adult Armenian hamsters (*Cricetulus migratorius*) were purchased from Cambridge Diagnostic (Cambridge, MA). All strains of mice used in this study were either obtained from The Jackson Laboratory or were bred in our own colony (National Institutes of Health, Bethesda, MD). Tumor cell lines used in this study included K562, a human erythroleukemia cell line, and 2B4, a cytochrome *c*-specific T-cell hybrid (21). Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum as described (21).

Monoclonal Antibodies and Antisera. The anti-Ly-6C (144-4B11) and anti-Thy-1 (145-7B10) mAbs generated in our laboratory (J.A.B. and M.F.) are of Armenian hamster origin (data not shown). Other mAbs used included 83-12-5 (anti-Lyt-2.2, generated in our laboratory), HO-13-4 (anti-Thy-1.2, ref. 22), H35-89.9 (anti-LFA-1, ref. 23), and RL 172.4 (anti-L3T4, ref. 24). The R9 antiserum (25), specific for the murine T3-δ chain, was generated by immunizing a rabbit with a C-terminal peptide synthesized according to the amino acid sequence deduced from the cDNA sequence (10).

Immunization and Fusion Protocols. Armenian hamsters were preferred over Syrian hamsters as recipients for immunizations because of the higher fusion efficiencies obtained with the murine myeloma SP2/0 (ref. 26 and M.F., unpublished observation). Adult female hamsters were first primed by an intradermal injection of an H-$2K^b$-specific murine cytotoxic T-lymphocyte (CTL) clone (10^7 cells; clone BM10-37, ref. 27) emulsified in complete Freund's adjuvant (Difco),

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Abbreviations: TCR, T-cell receptor; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody.

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given a booster injection 2 weeks later with the same dose of antigen in incomplete Freund's adjuvant, and injected every 2–3 weeks intraperitoneally with cloned cells in 0.9% NaCl, for a total of six injections. After resting for 2–3 months, selected animals were given an intravenous booster injection (2×10^7 cells) and killed 3 days later. Fusion 145 was performed as follows: the spleen was aseptically removed and a single-cell suspension was prepared; 2×10^8 spleen cells were mixed with 2.5×10^7 cells of the murine myeloma cell line SP2/0, fused according to published protocols (28), and distributed in 960 individual cultures. Growth of hybridoma cells was observed in 100% of the wells, suggesting that more than one clone was present in each culture. Supernatant of each culture was tested for its ability to induce the H-$2K^b$-specific CTL clone BM10-37 to lyse the H-$2K^b$-negative target cell K562, according to a protocol recently developed in our laboratory. We have recently shown (29) that antibodies to the TCR complex, including antibodies to murine TCR or anti-human T3 mAbs can redirect lysis of effector CTL to non-antigen-bearing, Fc receptor-positive targets. Anti-TCR complex antibodies probably trigger the lytic function by binding to TCR-complex structures on the effector population and provide cell–cell contact with the target cell through Fc receptor binding (29). Following this protocol, we cultured BM10-37 cells (10^4) for 16 hr at 37°C with ^{51}Cr-labeled ($Na^{51}CrO_3$, Amersham) Fc receptor-positive K562 cells (10^3) in the presence of 50 μl of culture supernatant from individual microcultures. The percent specific lysis was determined as previously published (27). Hybridoma culture supernatants were scored as positive when >10% specific lysis of the K562 target was observed.

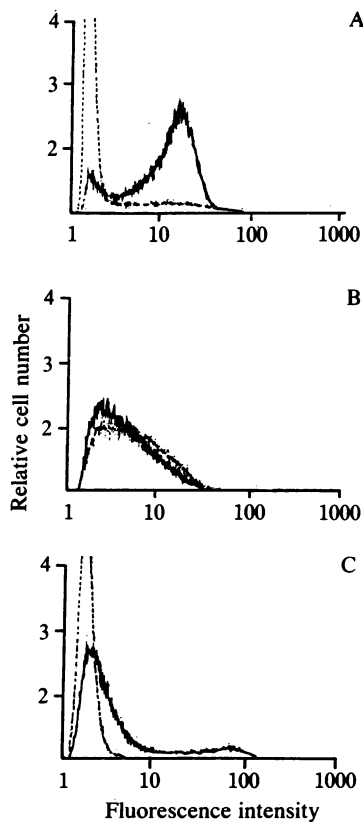

FIG. 1. Expression of the 145-2C11 marker on resting lymphoid cells. Cells were incubated with 145-2C11 (solid line) or control (dashed line) culture supernatant and counter-stained with a fluoresceinated anti-hamster immunoglobulin. The data are displayed as immunofluorescence profiles in which the logarithm of fluorescence intensity was plotted on the *x* axis, and cell number, on the *y* axis. (*A*) Splenic T cells. (*B*) Splenic B cells. (*C*) Thymic cells.

Flow Cytometric Analysis. Flow cytometry was performed as described (30), using a modified Becton Dickinson FACS II. Hamster antibodies were detected using a fluorescein isothiocyanate-conjugated anti-hamster immunoglobulin reagent (Cooper Biomedical, Malvern, PA). Cell separations were performed as follows. Splenic T cells (>95% Thy-1.2$^+$) were prepared by double passage over a rabbit anti-mouse immunoglobulin antibody-coated plate as described (31). Non-T cells (>95% surface immunoglobulin-positive) were obtained after treating spleen cells with an anti-Thy-1.2 mAb (HO-13-4, ref. 22) and rabbit complement.

Biochemical Analysis. Cell surface radioiodination of the 2B4 T-cell hybrid by the lactoperoxidase-catalyzed reaction was done as described (7). Immunoprecipitation was performed with complexes prepared by adsorbing the relevant antibody to protein A-coupled Sepharose beads (Pharmacia, Uppsala, Sweden). Solubilized precipitates were electrophoresed in 13% polyacrylamide gels as described (7). Mild dissociation of the complexes was achieved by incubating the washed, immunoprecipitated complexes with 50 mM Tris·HCl, pH 7.6/300 mM NaCl/0.2% $NaDodSO_4$/0.1% Triton X-100/0.2% sodium deoxycholate on ice for 30 min, and the dissociated complexes were analyzed by electrophoresis. For two-dimensional electrophoresis, immunoprecipitated complexes were eluted in the absence of reducing agents and

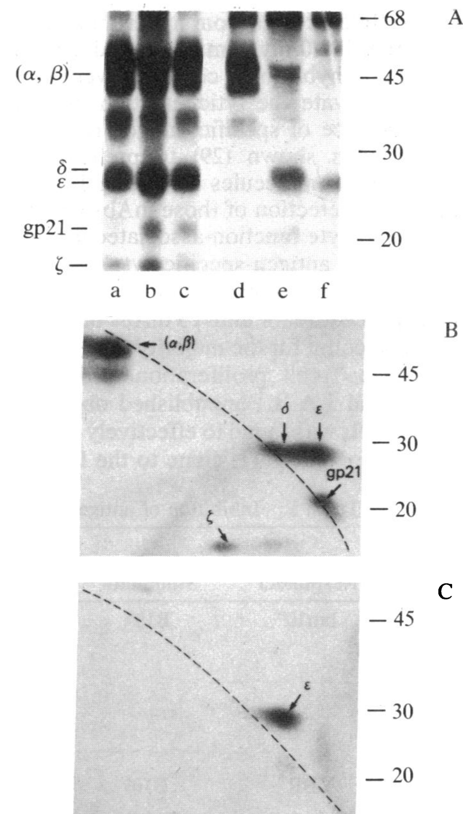

FIG. 2. Biochemical analysis of the 145-2C11-reactive protein. (*A*) Surface-radioiodinated 2B4 cells were immunoprecipitated with the anti-TCR mAb A2B4-2 (lanes a and d), the anti-T3-δ antiserum R9 (lanes b and e), and mAb 145-2C11 (lanes c and f). For lanes d–f, the immunoprecipitates were incubated with ionic detergents before electrophoresis as described in *Materials and Methods*. (*B*) Surface-labeled 2B4 cells were immunoprecipitated with mAb 145-2C11 and then subjected to two-dimensional (nonreducing/reducing) electrophoresis. (*C*) Immunocomplexes immunoprecipitated by mAb 145-2C11 were incubated with detergents before two-dimensional electrophoresis. In all panels, the different components of the immunoprecipitated TCR–T3 complex are identified by arrows. Molecular masses of standards are given in kDa at right.

electrophoresed in a 13% polyacrylamide tube gel (left to right in Fig. 2 *B* and *C*). After electrophoresis, the gel was equilibrated with 0.5% dithiothreitol in 0.1% $NaDodSO_4$ and the reduced proteins were electrophoresed in a slab gel as described (7).

***In Vitro* Assay Cultures.** Primary mixed lymphocyte reactions and CTL effector–function analyses were as described (27). The mitogenic properties of the mAbs were assessed by proliferation assays. T cells (10^5) were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 50 μM 2-mercaptoethanol with irradiated (2000 rads; 1 rad = 0.01 Gy) spleen cells (2×10^5) in the presence or absence of mAb and/or factors in flat-bottomed microwell plates. The recombinant human interleukin 2 was kindly provided by Cetus (Palo Alto, CA). After 2 days, the cells were incubated with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per well for 18 hr, the samples were harvested, and incorporation of radioactive isotope was measured with a scintillation counter.

RESULTS AND DISCUSSION

The purpose of this study was to generate mAbs specific for cell surface structures involved in murine T-cell activation. Armenian hamsters rather than rats were injected with a murine alloreactive cytolytic clone (BM10-37) to maximize the phylogenetic difference between responder and immunogen while retaining the ability to generate monoclonal antibodies (26). Splenic cells from hyperimmunized animals were fused to the SP2/0 myeloma line, and supernatants of cultures containing hybridoma cell lines were screened for their ability to activate the lytic function of murine CTL clones in the absence of specific antigen recognition. This procedure has been shown (29) to preferentially detect antibodies directed at molecules mediating T-cell activation while avoiding the detection of those mAbs that, by binding to Lyt-2 or lymphocyte function-associated antigen 1 (LFA-1) molecules, inhibit antigen-specific cytolysis. Induction of lytic activity was preferred over the induction of proliferation as a screening procedure for anti-T3 mAbs because anti-Thy-1 mAbs, which account for the majority of xenogeneic mAbs produced, induce T-cell proliferation (32) but not CTL function (O.L. and J.A.B., unpublished observation). One antibody, 145-2C11, was found to effectively redirect lysis by a murine H-2K^b-specific CTL clone to the H-2K^b-negative, Fc receptor-positive human target cell line K562. The hybrid cell line producing 145-2C11 was therefore subcloned by limiting dilution and used for further studies.

The expression of the 145-2C11-defined epitope on lymphoid tissues was studied by flow cytometry. The 145-2C11 antibody reacted with a subpopulation of lymphoid cells from all mouse strains examined but did not react with peripheral lymphocytes from rats, rabbits, miniature swine, or hamsters. Peripheral T cells but not B cells (Fig. 1) or bone marrow cells (data not shown) expressed this determinant. Although the expression of the 145-2C11 epitope on peripheral T cells was uniformly high, the staining of thymocytes revealed distinct subpopulations of cells differing in the level of expression of the 145-2C11 marker (Fig. 1). Preliminary studies identified the subpopulation of thymic cells expressing high levels of the 145-2C11 epitope as the phenotypically mature (L3T4$^+$,Lyt-2$^-$ and L3T4$^-$,Lyt2$^+$) thymocytes, whereas the remaining T cells express low but significant levels of the 145-2C11 antigen (J.A.B., B. J. Fowlkes, and S. Sharrow, unpublished results). Finally, all monoclonal T-cell lines tested, including many T-cell tumors and antigen-specific T-cell hybridomas, helper T cells, and CTL clones, expressed the 145-2C11 determinant (data not shown).

Immunoprecipitation studies were performed to determine whether 145-2C11 would react with a component of the TCR–T3 complex (Fig. 2). An antigen-specific T-cell hybrid, 2B4 (21), was surface-labeled with ^{125}I by the lactoperoxidase method and then solubilized with detergent. Aliquots of the radiolabeled material were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis under reducing conditions. The clone-specific anti-TCR antibody (A2B4-2, ref. 21) immunoprecipitated the α and β chains of the TCR (42–45 kDa), as well as five additional proteins of lower molecular mass comprising the murine T3 complex (Fig. 2*A*, lane a). The dense 25- to 26-kDa band contained two polypeptides, a 26-kDa endoglycosidase-sensitive glycoprotein (T3-δ) and a 25-kDa endoglycosidase-resistant protein, presently thought to represent the homologue of the human T3-ε chain. In addition, two bands at approximately 21 kDa and 16 kDa (gp21 and T3-ζ) were also apparent. A 36-kDa TCR protein was also observed, which may represent cleavage products of the heterodimer. As seen in Fig. 2*A* (lanes b and c), both a rabbit serum specific for the T3-δ chain (25) and the 145-2C11 mAb coprecipitated the α and β chains of the TCR hetero-

Table 1. Inhibition of antigen-specific lysis by mAb 145-2C11

			% specific lysis†		
Responder	Stimulator	mAb*	E/T 15:1	E/T 5:1	E/T 1.5:1
bm10‡	B10§	None	66.4	51.5	31.9
		anti-T3-ε (145-2C11)	6.7	5.5	1.9
		anti-Ly-6C (144-4B11)	70.1	56.9	38.3
		anti-Thy-1 (145-7E12)	79.2	55.8	31.8
		anti-Lyt-2.2 (83-12-5)	28.6	12.4	4.8
		anti-LFA-1 (H35-89.9)	2.2	−1.3	−2.1
YBR¶	B10§	None	48.8	35.5	20.2
		anti-T3-ε	−6.8	−6.7	−6.4
		anti-Ly-6C	59.0	42.8	25.5
		anti-Thy-1	54.0	33.9	17.5
		anti-Lyt-2.2	15.1	7.9	1.9
		anti-LFA-1	2.1	−3.1	−4.1

For inhibition of CTL activity, effector CTLs were preincubated 1 hr at 37°C with 50 μl of culture supernatant containing the mAb of interest, then cultured for an additional 4 hr with ^{51}Cr-labeled, antigen-bearing target cells. All antibodies were used at saturating concentrations. E/T, effector/target cell ratio.

*Name of each antibody is shown in parentheses.

†Underlined data indicate >90% inhibition of the specific lytic activity.

‡*H-2K^{bm10}, I-A^b, H-2D^b*.

§*H-2K^b, I-A^b, H-2D^b*.

¶*H-2K^b, I-A^b, H-2D^d*.

Table 2. Induction of non-antigen-specific lysis by mAb 145-2C11

Responder	Stimulator	mAb*	% specific lysis		
			E/T 100:1	E/T 33:1	E/T 10:1
bm10	B10	None	2.8	1.7	1.5
		anti-T3-ε	53.6	42.0	37.0
		anti-Ly-6C	1.0	0.6	−0.1
		anti-Thy-1	1.3	−0.1	0.1
		anti-Lyt-2.2	2.2	0.7	−0.6
		anti-LFA-1	0.1	0.5	0.5
YBR	B10	None	3.0	2.1	2.0
		anti-T3-ε	44.3	34.0	23.5
		anti-Ly-6C	3.7	2.9	2.1
		anti-Thy-1	3.5	0.8	1.4
		anti-Lyt-2.2	1.9	0.9	1.2
		anti-LFA-1	2.0	2.3	2.3

The induction of CTL activity was determined by incubating the effector CTL and the antigen-negative human target K562 in the presence of 5 μl of culture supernatant for 8 hr at 37°C. Similar results were obtained by using a wide range of antibody concentrations (from 50 μl to 1 μl of culture supernatant, data not shown). E/T, effector/target ratio.
*Designations of mAbs are given in Table 1.

dimer and the proteins of the T3 complex. Control antibodies, including culture supernatant from hybridoma lines secreting irrelevant hamster antibodies, failed to immunoprecipitate any material from the labeled cells (data not shown). In order to determine which polypeptide chain was specifically recognized by the 145-2C11 mAb, the immunoprecipitated complexes were dissociated by treatment with ionic detergents before electrophoresis (Fig. 2*A*, lanes d–f). Lane d shows that after detergent treatment, the two bands corresponding to the α and β subunits of the TCR are specifically retained in the A2B4-2 immunoprecipitate, indicating that this antibody binds to the TCR heterodimer. In contrast, both the R9 serum and the 145-2C11 mAb were specific for components of the murine T3 complex. A comparison of lanes e and f in Fig. 2*A* indicates that the R9 anti-T3-δ antiserum recognized the larger 26-kDa protein, whereas the 145-2C11 mAb recognized the smaller protein (T3-ε) in the dense 25- to 26-kDa band. The specificity of mAb 145-2C11 was confirmed by two-dimensional electrophoresis, which allowed a better resolution of the dense 25- to 26-kDa band observed in one-dimensional gels. Two-dimensional gel analysis of the 145-2C11 immunoprecipitate (Fig. 2*B*) resulted in a distinct migration pattern of proteins previously observed by ourselves and others using either anti-TCR antibodies (6) or the R9 antiserum (25). The antigen-specific TCR consisted of a disulfide-linked heterodimer (α and β) migrating below the diagonal. The disulfide-linked homodimer of the 16-kDa proteins (T3-ζ) is resolved below the diagonal; both the 26-kDa (T3-ζ) and the 21-kDa (gp21) subunits migrate along the diagonal; the fourth chain, the 25-kDa protein (T3-ε) runs above the diagonal with an apparent molecular mass of 22 kDa when not reduced and 25 kDa when reduced, suggesting the presence of intrachain disulfide bond(s). Dissociation by detergent treatment demonstrated that the mAb 145-2C11 reacts with T3-ε (Fig. 2*C*).

The functional properties of the anti-T3-ε mAb were also studied. Murine CTLs specific for the H-2K^b and H-2D^b molecules were generated during a primary allogeneic mixed lymphocyte reaction by culturing, respectively, B6.CH-2^{bm10} [bm10 (*H-2K^{bm10}*, *H-2D^b*)] or B10.YBR [YBR (*H-2K^b*, *H-2D^d*)] spleen cells with irradiated C57BL/10 [B10 (*H-2K^b*, *H-2D^b*)] lymphocytes. As shown in Table 1, mAb 145-2C11 inhibited the lysis of the target cells by the antigen-specific CTL effectors. Hamster mAbs specific for other murine T-cell surface markers expressed by CTLs, such as Ly-6C (144-4B11) or Thy-1 (145-7E12), did not inhibit CTL activity.

The ability of the 145-2C11 mAb to activate murine T cells is shown in Tables 2 and 3. The ability of mAbs to redirect lysis of murine CTLs to irrelevant target cells was assessed. As expected, none of the alloreactive murine CTLs signifi-

Table 3. Induction of T-cell proliferation by mAb 145-2C11

Exp.	Responder*		PMA‡	rIL-2§	[^{3}H]Thymidine incorporated, cpm × 10^{-3}	
	Strain	Treatment†			Control mAb¶	145-2C11
1	B10	None	−	−	1.0	61.5
			+	−	6.2	249.6
			−	+	5.2	258.0
2	B10	C′	−	+	2.6	90.4
		anti-Lyt-2 + C′	−	+	3.9	161.6
		anti-L3T4 + C′	−	+	7.0	57.9
		C′	+	−	3.1	137.1
		anti-Lyt-2 + C′	+	−	5.2	128.3
		anti-L3T4 + C′	+	−	6.9	54.3

*C57BL/10 T cells (10^5) were cultured with irradiated spleen cells (2×10^5) as stimulators.
†Spleen cells were incubated with antibodies to the Lyt-2 or L3T4 molecules followed by rabbit complement (C′). In both cases, antibody treatment resulted in the elimination of >95% of the corresponding cell population, as judged by flow cytometry (data not shown).
‡Phorbol 12-myristate 13-acetate (10 ng/ml).
§Recombinant interleukin 2 (50 units/ml).
¶mAb 145-8B10.

cantly lysed the human target cell K562 in the absence of mAb (Table 2). However, addition of culture supernatant from the hybridoma secreting the anti-T3-ε mAb resulted in the lysis of target cells. The lysis was dependent on the presence of effector CTLs, as incubation of the K562 cells with the mAb alone did not result in target-cell lysis (data not shown). None of several other antibodies, including anti-Lyt-2.2 and anti-LFA-1 mAbs, that inhibited antigen-specific lytic activity (see Table 1) were able to promote lysis of the K562 targets by the CTL effectors. This ability to redirect lysis of the BM10-37 CTL clone to the K562 target had in fact been used as the screening procedure for identifying the 145-2C11 mAb (data not shown).

The ability of mAb 145-2C11 to induce T-cell proliferation was also studied (Table 3). Purified T cells were cultured with 145-2C11 or control mAbs in the absence or presence of costimulating factors. The 145-2C11 mAb induced significant proliferation in the absence of exogenous factors. However, the addition of either phorbol 12-myristate 13-acetate or recombinant interleukin 2 significantly increased the proliferative response. Finally, both Lyt-2^+,L3T4^- and Lyt2^-, L3T4^+ splenic T cells proliferated in the presence of soluble anti-T3-ε mAb (Table 3, Exp. 2). By comparison, a soluble anti-V_β8-specific mAb (F23.1), has been shown to strongly stimulate Lyt-2^+ T cells but to only minimally affect L3T4^+ lymphocytes (ref. 33; K. Hathcock and R. J. Hodes, personal communication). Thus, phenotypically distinct subsets of T cells might be differentially activated by antibodies specific for different components of the murine TCR–T3 complex.

CONCLUSIONS

Although T3 components can be analyzed extensively in humans, some analyses, including the expression of T3 in T-cell ontogeny and development, and *in vivo* treatment, could more readily be performed in the mouse model. This has not been possible due to the lack of mAbs to the murine T3 polypeptides. mAb 145-2C11, described and characterized in this study, makes such studies possible. This mAb is specific for the 25-kDa T3 protein tentatively identified as T3-ε. Functional analyses revealed that the anti-murine T3-ε mAb can serve as either an agonist or an antagonist in T-cell activation. These data extend the observations previously made in murine and human systems with antibodies to the TCR complex and indicate that antibodies directed at different components of this complex—namely, the TCR $\alpha\beta$ heterodimer, the T3-δ chain, or the T3-ε chain—share similar functional properties. Thus, this mAb will be useful in studying the role of various components of the TCR complex in T-cell activation and development, as well as the expression of other TCR gene products (for example, the γ gene) associated with T3 polypeptides, and the mAb will allow the development of an animal model in which to investigate the immunoregulatory effects of *in vivo* administration of anti-T3 antibodies, an area of obvious clinical importance.

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