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Effects of Cytotoxic Monoclonal Antibody Specific for T200 Glycoprotein on Functional Lymphoid Cell Populations[1]

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A monoclonal antibody against T200 glycoprotein is selectively cytotoxic for thymocytes and mature thymus-dependent (T) lymphocytes. All T-cell functions assayed, cell-mediated cytotoxicity, helper cell activity, and proliferation in response to T-cell mitogens or allogeneic cells were abolished by prior treatment of spleen cells with anti-T200 antibodies and complement. In contrast, thymus-independent (B) cell responses to lipopolysaccharide (LPS) and other B-cell mitogens were unaffected. Although treatment of spleen cells with anti-T200 antibodies and complement markedly reduced their capacity to mount an *in vitro* antibody response to sheep red blood cells (SRBC), responsiveness could be restored by the addition of SRBC-primed T-helper cells. Treatment of bone marrow cells with anti-T200 antibodies and complement did not eliminate either *in vivo* colony-forming units-spleen (CFU-S) or prothymocytes. It is concluded that T lymphocytes become sensitive to complement-mediated lysis by anti-T200 antibodies as a consequence of cell-surface modifications occurring shortly before or just after their entry into the thymus. In contrast to Thy-1 antigen, the selective killing by antibody against T200 glycoprotein cannot be readily accounted for by quantitative differences in the expression of T200 glycoprotein on the cell surface. Fluorescence-activated cell analysis showed that T200 glycoprotein was expressed in similar amount on the majority of all thymocytes, spleen, and bone marrow cells.

INTRODUCTION

T200 glycoprotein is a major cell-surface glycoprotein found on murine thymocytes and T[2] cells, and antigenically indistinguishable glycoproteins are widely distributed on murine hematopoietic cells and their tumors ((1–3) R. Hyman, I. Trowbridge, and K. Cunningham, *J. Cell. Physiol.*, in press). Similar glycoproteins have been identified on rat leukocytes (4). In both species these molecules appear to be restricted to hematopoietic cells within the limits of detection of the quantitative absorption assays employed. Recently we have obtained a monoclonal antibody against T200 glycoprotein (3). Here we report that this monoclonal antibody is

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[2] Abbreviations used: B, bone marrow-derived; BSS, Hanks' balanced salt solution; Con A, concanavalin A; CFU-S, colony-forming unit-spleen; C', complement; FITC, fluorescein isothiocyanate; H–DMEM, Hepes-buffered Dulbecco's modified Eagle's medium; LPS, bacterial lipopolysaccharide; PHA, phytohemagglutinin; PPD, purified tuberculin; PWM, pokeweed mitogen; SRBC, sheep red blood cells; T, thymus derived.

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selectively cytotoxic for thymocytes and mature T cells. The selective cytotoxicity of the anti-T200 antibody was strikingly similar to that of monoclonal antibodies against Thy-1 glycoprotein tested in parallel experiments. However, in contrast to anti-Thy-1 antibodies, the selective killing of thymocytes and mature T cells by anti-T200 antibodies cannot readily be accounted for by quantitative differences in the expression of T200 glycoprotein on different classes of hematopoietic cells.

MATERIALS AND METHODS

Animals and cell lines. C57BL/6 and BALB/c mice were obtained from the Frederick Cancer Research Center (Frederick, Md.). BALB/c (Nu/Nu) mice were obtained from Sprague–Dawley, AKR/J, and (BALB/c × A/J)F_1 mice from Jackson Laboratories, and AKR/Cum from Cumberland View Farms (Tenn.). BW5147 (Thy-1^+) and S194/2 cell lines were maintained in Dulbecco's modified Eagle's minimal essential medium supplemented with either 10% fetal calf serum or horse serum.

Antibodies and serological procedures. Rat spleen cell–mouse myeloma hybrids producing antibodies against T200 glycoprotein (I3/2.3) and Thy-1 glycoprotein (three independent hybrids designated C22/22.7.1.1., T24/31.7, and T24/40.7) were obtained essentially as described previously (3). The monoclonal antibodies against Thy-1 glycoprotein were characterized by immunoprecipitation studies, quantitative absorption tests with various mouse tissues, and by direct binding assays using mutant Thy-1^- and wild-type Thy-1^+ cell lines. All three antibodies precipitated Thy-1 glycoprotein from detergent extracts of Thy-1^+ cells and did not discriminate between Thy-1.1 and Thy-1.2 alloantigenic determinants (I. S. Trowbridge, unpublished results). Unless stated otherwise, the anti-Thy-1 monoclonal antibody used was C22/22.7.1.1. Culture supernatants pooled from heavy cultures of hybrid cells provided the source of antibody. The same preparation of each antibody was used for all experiments. Anti-Thy-1.1 alloantisera were obtained from AKR/Cum mice immunized with AKR/J thymocytes, and anti-Thy-1.2 alloantibodies were raised by immunizing AKR/J mice with C3H/HeJ thymocytes.

Direct cytotoxic tests and quantitative cytotoxic absorptions were carried out as described (5, 6). Red blood cells were removed from spleen and bone marrow cell suspensions by treatment with Tris-buffered ammonium chloride (7). The complement source was guinea pig serum diluted 1:3 in Hepes-buffered Dulbecco's modified Eagle's medium (H-DMEM) for all assays in which alloantisera were used. A rabbit serum selected for low toxicity was routinely used at a final dilution of 1:24 for assays in which monoclonal antibodies were employed. Spleen cells to be treated with antibody for functional assays were suspended at 2×10^7 cells/ml in Hank's balanced salt solution (BSS) containing various concentrations of antibody for 30 min at 4°C. The supernatant was removed after centrifugation and an equal amount of BSS containing a 1:20 final dilution of rabbit complement was added. After incubation for 45 min at 37°C, the cells were washed three times in BSS and used for *in vitro* assays.

Mitogenic stimulation and mixed lymphocyte reaction. Both assays were performed in Falcon microtiter plates (No. 3040) in quadruplicate and harvested by an automated multiharvester (Otto Hiller, Madison, Wis.). Spleen cells were

cultured in a total volume of 220 μl/well in Eagle's MEM for suspension cultures (Microbiological Associates, Bethesda, Md.), supplemented with 10% fetal calf serum and 5×10^{-5} M β-mercaptoethanol. Responder cells (3×10^5) were either mixed with mitogen or 6×10^5 allogeneic spleen cells previously irradiated with 1000 R from a ^{60}Co source. The following mitogens were used: phytohemagglutinin (PHA) (Gibco, Grand Island, N.Y.), pokeweed mitogen (PWM) (Gibco) concanavalin A (Con A) (Miles Labs., Elkhart, Ind.), *Escherichia coli* lipopolysaccharide (LPS) (Difco, Detroit, Mich.), and purified tuberculin (PPD) (Statens Serum Institute, Copenhagen, Denmark). All mitogens were used at previously determined optimal concentrations. At 24-hr intervals, cell proliferation was assayed by pulse-labeling the cultures in 20 μl of a mixture containing 0.5 μCi [^{3}H]thymidine (sp act 5 Ci/mmol), 10^{-5} *M* unlabeled thymidine, and 10^{-6} *M* fluorodeoxyuridine. After 20 hr, the cells were harvested on glass-fiber filters, and the radioactivity incorporated into trichloroacetic acid-insoluble material was determined.

Induction of cytotoxic cells and plaque-forming cells. Induction of cytotoxic cells was carried out by culturing spleen cells (10^7 cells/ml/dish) with allogeneic spleen cells previously irradiated with 1000 R (5×10^6 cells/ml/dish) in 3-cm-diameter tissue culture dishes in RPMI 1640 medium (Gibco). This medium was supplemented with 5% FCS, glutamine (final concentration 5 m*M*), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 5×10^{-5} *M* mercaptoethanol. The cultures were incubated in plastic boxes (5% CO_2, 95% air) at 37°C under constant rocking (8). Cells were harvested on day 5, at which time, under these culture conditions, maximal cytolytic activity is seen and immediately assayed for cytotoxic activity.

For the induction of an *in vitro* response, spleen cells (10^7/ml) were cultured for 5 days with 10^6 sheep red blood cells (SRBC) per milliliter in 3-cm tissue culture dishes in RPMI 1640 medium. The number of plaque-forming cells (PFC) was assayed on SRBC using a modification of the Jerne plaque assay on microscope slides (8).

Cytotoxicity assay. Spleen cells and ^{51}Cr-labeled target cells were incubated at various ratios of spleen cell attacker to ^{51}Cr-labeled target cells (a/t) in RPMI 1640 medium containing 10% FCS at 37°C. To prepare labeled lymphoid target cells, 5×10^6 cells were incubated in 1 ml of RPMI 1640 medium with 10% FCS and 100–200 μCi ^{51}Cr for 30 min at 37°C, the cells were then washed three times in Hanks' balanced salt solution (BSS) containing 10% FCS. Groups of tubes containing 2×10^7 spleen cells and 2×10^5 labeled target cells, for a spleen to target cell ratio of 100:1 (for lower ratios the number of spleen cells was reduced) in a total volume of 2 ml were set up and 0.25-ml aliquots were dispensed into Falcon plastic tubes (No. 2057), flushed with 5% CO_2 in air, stoppered, and incubated at 37°C on a rocking platform (Bellco Glass, Vineland, N.Y.). At specified times, 1.75 ml BSS was added per tube, followed by mixing and centrifugation for 5 min at 1000*g*. Samples (1 ml) of supernatants were counted in an automatic well-type gamma radiation counter. Cytotoxicity was calculted as

$$\% \text{ cytotoxicity} = \frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximal } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100.$$

Maximal release was determined by freezing and thawing the target cells three times.

Spleen colony assay for CFU-S. (BALB/c × A/J) bone marrow at 5×10^6 cells per tube was treated with 0.5 ml of tissue culture supernatant containing monoclonal antibody diluted 1:4 in H–DMEM and 0.5 ml rabbit complement diluted 1:10 in H–DMEM. Controls had H–DMEM substituted for monoclonal antibody or complement. After incubation at 37°C for 45 min (Expt 1) or 80 min (Expt 2), the cells were centrifuged, washed once, and suspended at 2×10^6/ml in H–DMEM. (BALB/c × A/J) mice irradiated with 850–900 *R* from a ^{60}Co source were injected with 4×10^5 cells iv. Spleens were removed 9 days later, fixed overnight in Bouin's solution, and the macroscopic spleen colonies were counted (9).

Prothymocyte assay. The assay for prothymocytes was based on the previous work of Kadish and Basch (10). Bone marrow cells from 4-week-old AKR/J mice were treated with monoclonal antibody (final dilution 1:8 in H–DMEM) and rabbit complement (final dilution 1:20) at 5×10^6 cells/ml for 45 min at 37°C. Cells were then centrifuged, washed once, and resuspended to 2.5×10^7 cells/ml. AKR/Cum mice, 6 weeks old, were irradiated with 780 R (87 R/min from a ^{60}Co source) immediately before iv injection of 0.2 ml (5×10^6 cells) of the AKR/J bone marrow cell suspension. The proportion of thymocytes bearing either Thy-1.1 or Thy-1.2 was determined for individual recipient mice in triplicate by direct cytotoxicity testing 10, 17, and 24 days later.

Fluorescence-activated cell analysis. Binding of T200 and Thy-1 antibodies to lymphoid cell populations was determined by quantitative indirect fluorescence using a Los Alamos-type fluorescence-activated cell analyzer. Red blood cells were removed from normal lymphoid cell populations by treatment with Tris-buffered ammonium chloride. Cells (5×10^6) in 0.2 ml of 0.15 *M* NaCl–0.015 *M* NaN_3–0.01 *M* sodium phosphate buffer (pH 7.2) were incubated on ice for 45 min with 0.2 ml of the appropriate antibody. After extensive washing the cells were then incubated on ice for 45 min with 0.2 ml of fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-rat IgG antibodies (50 μg/ml) from which antibodies reactive with mouse IgG had been previously removed by passage through a column of mouse IgG-Sepharose. After washing, cells were examined on the fluorescence-activated cell analyzer to determine the percentage of fluorescent cells and their relative staining using appropriately gated light scatter to determine the total number of cells analyzed.

RESULTS

Cytotoxicity of Anti-T200 Antibody for Murine Hematopoietic Cells

Direct cytotoxicity tests showed that over a wide range of antibody concentrations anti-T200 monoclonal antibody in the presence of complement killed more than 95% of thymocytes but only about 40% of spleen cells and 20–30% of bone marrow cells (Fig. 1A). The partial killing of spleen and bone marrow does not appear to be a specific property of our anti-T200 monoclonal antibody, since another anti-T200 antibody (30-F11, kindly provided by Dr. J. Ledbetter, Stanford Medical School, Ref. (11)) also killed a similar fraction of spleen cells (Fig. 1B). Both T200 antibodies are of the $IgG2_b$ subclass (3, 11). In other experiments, anti-T200 antibody in the presence of complement killed about 15–20% of spleen cells from BALB/c (Nu/Nu) mice. In all these experiments, killing by complement alone was about 10% of the cells.

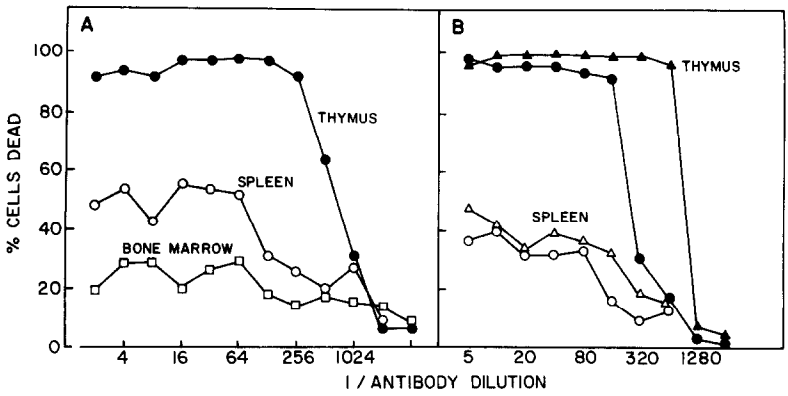

FIG. 1. Direct cytotoxicity of Anti-T200 antibody. (A) shows the percentage of cells killed from thymus, spleen, and bone marrow by anti-T200 (I3/2.3) monoclonal antibodies. (B) shows a comparison of the percentage of cells killed from thymus and spleen by two different anti-T200 monoclonal antibodies (I3/2.3: ●, ○; and 30-F11. ▲, △).

When anti-T200 and anti-Thy-1 monoclonal antibodies were tested in parallel, anti-Thy-1 antibodies usually killed about 20–30% of spleen cells and 10–20% of bone marrow cells, whereas anti-T200 killed 30–40% of spleen cells and 15–25% of bone marrow cells. The same results were obtained with different batches of rabbit complement used at several different concentrations. In order to test whether anti-Thy-1 and anti-T200 antibodies were killing different or overlapping populations of spleen cells, the proportion of cells killed by treatment of the two antibodies together or by sequential treatment with each antibody was measured. As shown in Table 1, treatment of spleen cells with both anti-T200 and anti-Thy-1 antibodies together did not increase the proportion of cells killed above the values obtained when spleen cells were incubated with complement and each antibody separately. Furthermore, spleen cells surviving treatment with either anti-T200 antibodies or anti-Thy-1 antibodies were not susceptible to lysis by subsequent treatment with the reciprocal antibody.

Effects of Cytotoxic Ablation with Anti-T200 Antibodies on the Responses of Spleen Cells in in Vitro Functional Assays

The results described in the previous section suggest that the majority of spleen cells susceptible to lysis by anti-T200 antibodies and complement express Thy-1 and therefore are probably T cells. To examine this question in more detail, the effects of prior treatment of spleen cells with anti-T200 antibodies and complement on T- and B-cell functions were studied. As shown in Table 2, prior treatment of spleen cells with anti-T200 antibodies and complement completely abolished the proliferative responses to the T-cell mitogens, PHA and Con A, but had no effect on the stimulation of spleen cells by either LPS or PPD, B-cell mitogens, even at a concentration at least fivefold higher than that required to completely abrogate responses to T-cell mitogens. Both T-killer cells and their precursors were sensitive to anti-T200 antibodies and complement (Table 3), as shown by the complete abrogation of cytolytic activity by treatment either before or after induction of cytotoxic cells. Similarly, the proliferative response of spleen cells to allogeneic

TABLE 1

Treatment of Spleen Cells with Anti-T200 and Anti-Thy-1 Monoclonal Antibodies

First treatment[a]	Second treatment	Percentage spleen cells dead[b]
C′		8 ± 2
α-T200 + C′		33 ± 4
α-Thy-1 + C′		26 ± 1
α-T200 + α-Thy-1 + C′		26 ± 2
C′	C′	8 ± 3
	α-T200 + C′	30 ± 5
	α-Thy-1 + C′	31 ± 1
	α-T200 + α-Thy-1 + C′	29 ± 4
α-T200 + C′	C′	8 ± 1
	α-T200 + C′	9 ± 1
	α-Thy-1 + C′	10 ± 2
	α-T200 + α-Thy-1 + C′	11 ± 2
α-Thy-1 + C′	C′	13 ± 4
	α-T200 + C′	15 ± 2
	α-Thy-1 + C′	12 ± 3
	α-T200 + α-Thy-1 + C′	17 ± 1
α-T200 + α-Thy-1 + C′	C′	5 ± 1
	α-T200 + C′	6 ± 2
	α-Thy-1 + C′	7 ± 1
	α-T200 + α-Thy-1 + C′	9 ± 1

[a] Spleen cells were prepared by removal of red blood cells by lysis with Tris-buffered ammonium chloride followed by centrifugation over Ficoll-Hypaque (Lympholyte-M, Cedarlane Labs). After the first incubation, dead cells were removed by centrifuging over Ficoll–Hypaque. Cell recoveries ranged from 50 to 70%.

[b] Average ± SE of triplicate determinations.

cells was almost completely abrogated by prior treatment of the responder cells with anti-T200 antibodies and complement (Table 4). The effect of ablation of anti-T200 sensitive cells on an *in vitro* anti-SRBC antibody response was also investigated. It can be seen from Table 5 that at the highest concentration of anti-T200 antibody used, the anti-SRBC response was completely inhibited. However, since the antibody response was restored by the addition of SRBC-primed T cells (Expt 2, Table 5), it was probable that the observed abrogation of the antibody response was the result of the elimination of T helper cells. This was confirmed by mixing spleen cells treated with monoclonal anti-Thy-1 serum with various numbers of T helper cells treated with different concentrations of anti-T200 antibody (Table 6). Prior treatment of the T helper cell population with either anti-T200 antibody or anti-Thy-1 antibody completely abolished the helper activity of the T-cell population.

Prothymocyte and CFU-S Assays: Pretreatment of Bone Marrow Cells with Anti-T200 Antibodies

We examined whether the hematopoietic precursors of thymocytes were also sensitive to complement-mediated lysis by anti-T200 antibodies to determine

TABLE 2

Effect of Prior Treatment of Spleen Cells with Anti-T200 Antibody and Complement on B- and T-Cell Mitogen Responses

Treatment[a]	Mitogen	[^{3}H]TdR incorporation[b] Day 1	Day 2
—	—	361 ± 61	525 ± 68
C′	—	410 ± 31	726 ± 36
—	5 μg/ml PHA	9,025 ± 417	7,180 ± 1502
C′	5 μg/ml PHA	13,351 ± 680 (33)	9,494 ± 556 (13)
α-T200 1:2 + C′	5 μg/ml PHA	508 ± 97 (1.2)	849 ± 50 (1.2)
α-T200 1:10 + C′	5 μg/ml PHA	597 ± 32 (1.5)	593 ± 186 (<1)
—	10 μg/ml Con A	31,271 ± 2182	39,808 ± 763
C′	10 μg/ml Con A	54,624 ± 2553 (133)	59,927 ± 403 (83)
α-T200 1:2 + C′	10 μg/ml Con A	840 ± 33 (2)	480 ± 15 (<1)
α-T200 1:10 + C′	10 μg/ml Con A	934 ± 17 (2.3)	644 ± 54 (<1)
—	5 μg/ml LPS	4,056 ± 483	6,473 ± 365
C′	5 μg/ml LPS	12,813 ± 821 (31)	12,620 ± 2266 (17)
α-T200 1:2 + C′	5 μg/ml LPS	12,880 ± 1061 (31)	12,354 ± 169 (17)
α-T200 1:10 + C′	5 μg/ml LPS	12,737 ± 692 (31)	10,908 ± 247 (15)
—	100 μg/ml PPD	2,891 ± 226	1,537 ± 77
C′	100 μg/ml PPD	3,892 ± 218 (9.5)	3,329 ± 195 (4.6)
α-T200 1:2 + C′	100 μg/ml PPD	3,040 ± 32 (7.4)	1,770 ± 55 (2.4)
α-T200 1:10 + C′	100 μg/ml PPD	2,644 ± 118 (6.4)	1,741 ± 170 (2.4)

[a] C57BL/6 spleen cells were treated as described before incubation with mitogens. See Materials and Methods for further details. C′-Treated spleen cells showed consistently higher responses, as is usual for this type of experiment.

[b] Average value ± SE, stimulation factors are given in parentheses.

whether this could provide a means of distinguishing between various classes of progenitor cells. The ability of bone marrow cells to form colonies in the spleens of irradiated recipients or to repopulate the thymus was used to assay for CFU-S and prothymocytes, respectively (9, 10). As shown in Table 7, pretreatment of bone marrow cells with either anti-T200 or anti-Thy-1 antibodies had little or no effect on the number of spleen cell colonies formed in irradiated recipients. In these experiments, RMB 30, a monoclonal antibody against mouse brain (R. Hyman, unpublished results) was used as a positive control and caused a highly significant reduction in the number of spleen colonies obtained.

The results of one of two assays for prothymocytes which gave similar results are shown in Fig. 2. If prothymocytes are sensitive to complement-mediated lysis by antibodies against T200 but CFU-S are not, it might be expected that removal of prothymocytes might result in a delay in repopulation of the thymus of recipient mice rather than a complete failure to repopulate since the prothymocyte population would be expected to eventually be restored from the pluripotent donor stem cells repopulating the recipients' bone marrow. For this reason we examined both the kinetics and the extent of thymic repopulation. The results of Kadish and Basch (10) show that the fraction of donor cells in the thymus increases from low

TABLE 3

Ablation of T-Killer Cells and Their Precursors by Complement-Mediated Lysis with Anti-T200 Monoclonal Antibody

Treatment	a/t	Percentage cytotoxicity	Percentage cytotoxicity
Expt 1. Before induction[a]		At 1.75 hr	
None	40:1	74 ± 4.0	
None	13:1	52 ± 4.0	
None	4:1	17 ± 0.5	
C′	40:1	60 ± 1.2	
C′	13:1	33 ± 0.1	
C′	4:1	13 ± 1.6	
α-T200 1:2 + C′	40:1	<1	
α-T200 1:8 + C′	40:1	<1	
α-T200 1:24 + C′	40:1	<1	
Expt 2. After induction		At 1.5 hr	At 3 hr
None	30:1	40 ± 0.5	65 ± 0.1
None	10:1	21 ± 0.5	42 ± 0.3
None	3:1	<1	7 ± 1.0
C′	30:1	42 ± 1.0	68 ± 0.4
C′	10:1	23 ± 1.0	50 ± 0.3
C′	3:1	<1	7 ± 2.0
α-T200 1:2 + C′	30:1	<1	<1
α-T200 1:10 + C′	30:1	<1	<1

[a] C57BL/6 spleen cells were treated either before (Expt 1) or after (Expt 2) induction of cytotoxic cells against BALB/c spleen cells. Cytotoxicity assays were then carried out using S194/2 BALB/c myeloma cells as the targets.

levels on Day 10 to almost 100% after 3–4 weeks so that by assaying at Days 10, 17, and 24 we would be able to detect both differences in the rate and extent of thymic repopulation. Our results are in complete agreement with these previous findings: on Day 10, few cells were found in the thymus and all were of host origin (data not

TABLE 4

Effect of Prior Treatment of Spleen Cells with Anti-T200 Antibody and Complement on Responsiveness in Mixed Lymphocyte Culture

Responder[a] spleen cells	Treatment	Stimulator spleen cells	[^{3}H]TdR incorporation Day 3
None	None	BALB/c	204 ± 49[b]
C57BL/6	None	None	390 ± 50
C57BL/6	None	BALB/c	3138 ± 533
C57BL/6	C′	BALB/c	3969 ± 315
C57BL/6	α-T200 1:2 + C′	BALB/c	689 ± 98
C57BL/6	α-T200 1:8 + C′	BALB/c	707 ± 64
C57BL/6	α-T200 1:24 + C′	BALB/c	1125 ± 237

[a] See Materials and Methods for additional experimental details.
[b] Average value ± SE.

TABLE 5

Effect of Prior Treatment of Spleen Cells with Anti-T200 Antibodies and Complement on *in Vitro* Anti-SRBC Response

Treatment	T helper cells[a]	SRBC	PFC/10^6 Recovered cells Day 5
Expt 1			
None	−	−	110
None	−	+	596
C′	−	+	516
α-T200 1:2 + C′	−	+	93
α-T200 1:8 + C′	−	+	200
α-T200 1:24 + C′	−	+	178
Expt 2			
None	−	−	6
None	−	+	250
C′	−	+	190
C′	+	+	1230
α-T200 1:2 + C′	−	+	72
α-T200 1:8 + C′	−	+	230
α-T200 1:24 + C′	−	+	150
α-T200 1:2 + C′	+	+	4800
α-T200 1:8 + C′	+	+	5150
α-T200 1:24 + C′	+	+	3400

[a] 5×10^6 cells were added per culture. T helper cells were prepared by injecting (iv) 5×10^7 syngeneic thymocytes and 0.2 ml of a 10% (v/v) suspension of SRBC into irradiated (1000 R) recipients. Spleen cells were harvested 7 days later and used as T helper cells. See Materials and Methods for additional experimental details.

shown). As shown in Fig. 2, by Day 17, partial thymic repopulation with cells of donor origin had occurred to various degrees and by Day 24 the thymuses of all recipient mice were fully repopulated with cells expressing the Thy-1 alloantigen of the donor type. It can be seen that neither treatment with antibodies against T200 or Thy-1 glycoproteins in the presence or absence of complement has a significant effect on either the rate or degree of repopulation with donor cells. Despite the failure to deplete prothymocytes by treatment with anti-T200 antibodies and complement, all the thymocytes of donor origin found in all the groups of recipient mice on Day 24 are sensitive to lysis by anti-T200 antibodies confirming that the progeny of the prothymocytes are susceptible to lysis by anti-T200 antibodies (data not shown).

Quantitative Expression of T200 and Thy-1 Glycoproteins on Thymus, Spleen, and Bone Marrow Cells

Although anti-T200 antibodies only kill a fraction of spleen and bone marrow cells, previous quantitative absorption studies showed that spleen and thymus contained about the same amount of T200 glycoprotein and bone marrow about half this amount (3). As shown in Fig. 3, quantitative serological absorption studies revealed that spleen cells from BALB/c (Nu/Nu) mice also expressed the same amount of T200 glycoprotein as spleen and thymus even though anti-T200

antibodies kill fewer than 10% of spleen cells from the athymic mice above control values. The quantitative expression of T200 glycoprotein on thymus, spleen, and bone marrow cells was investigated in more detail by fluorescence-activated cell

TABLE 6

Effect of α-T200 and α-Thy-1 on T Helper Cells

B cells ($\times 10^6$)	T helper cells[a]	Treatment	SRBC	Controls	PFC/10^6 Recovered cells on Day 5	
					α-T200	α-Thy-1
5	—	—	–	43		
5	—	—	+	20		
5	3×10^4	—	+	95		
5	10^5	—	+	357		
5	3×10^5	—	+	875		
5	10^6	—	+	1600		
5	3×10^6	—	+	2730		
5	3×10^4	C′	+	132		
5	10^5	C′	+	324		
5	3×10^5	C′	+	1080		
5	10^6	C′	+	2650		
5	3×10^6	C′	+	2247		
5	3×10^4	C′ + 1:5 antibody[b]	+		63	52
5	10^5	C′ + 1:5 antibody	+		104	62
5	3×10^5	C′ + 1:5 antibody	+		30	57
5	10^6	C′ + 1:5 antibody	+		128	73
5	3×10^6	C′ + 1:5 antibody	+		75	36
5	3×10^4	C′ + 1:15 antibody	+		130	90
5	10^5	C′ + 1:15 antibody	+		47	22
5	3×10^5	C′ + 1:15 antibody	+		52	71
5	10^6	C′ + 1:15 antibody	+		45	47
5	3×10^6	C′ + 1:15 antibody	+		38	58
5	3×10^4	C′ + 1:45 antibody	+		170	93
5	10^5	C′ + 1:45 antibody	+		160	53
5	3×10^5	C′ + 1:45 antibody	+		150	158
5	10^6	C′ + 1:45 antibody	+		240	541
5	3×10^6	C′ + 1:45 antibody	+		375	500
5	3×10^4	C′ + 1:135 antibody	+		50	108
5	10^5	C′ + 1:135 antibody	+		40	131
5	3×10^5	C′ + 1:135 antibody	+		344	95
5	10^6	C′ + 1:135 antibody	+		480	60
5	3×10^6	C′ + 1:135 antibody	+		490	275
5	3×10^4	C′ + 1:400 antibody	+		333	42
5	10^5	C′ + 1:400 antibody	+		400	113
5	3×10^5	C′ + 1:400 antibody	+		1333	180
5	10^6	C′ + 1:400 antibody	+		1190	280
5	3×10^6	C′ + 1:400 antibody	+		1000	950

[a] T helper cells were obtained from spleen cells of mice (injected iv 5 days previously with 10^6 SRBC) by filtration through nylon columns.

[b] Either anti-T200 or anti-Thy-1 antibodies.

TABLE 7

Assay for CFU-S after Treatment of Donor Bone Marrow with Monoclonal Antibodies Directed against T200, Thy-1, or the mouse brain determinant RMB30 and Complement

Treatment	Mean number of colonies ± SE	
	Expt 1	Expt 2
None	13.5 ± 3.0 (4)[a]	46.6 ± 2.2 (5)
Complement only	21.3 ± 4.1 (3)	44.3 ± 2.2 (6)
Anti-T200	19.2 ± 4.5 (5)	56.5 ± 3.5 (2)
Anti-T200 + complement	16.5 ± 3.4 (4)	33.8 ± 3.4 (6)
Anti-Thy-1	13.0 ± 2.6 (3)	45.2 ± 5.0 (6)
Anti-Thy-1 + complement	10.8 ± 2.8 (5)	35.8 ± 3.2 (5)
RMB30[b]	21.6 ± 3.5 (5)	52.7 ± 5.0 (3)
RMB30 + complement	3.4 ± 1.0 (5)	9.8 ± 1.7 (4)
No bone marrow	0.25 ± 0.3 (4)	0.0 (6)

[a] Numbers in parentheses are the number of surviving animals/group.

[b] RMB30 is a rat monoclonal antibody produced by a rat spleen–mouse myeloma hybrid cell line obtained from fusion of spleen cells immunized with mouse brain homogenate.

analysis (Fig. 4). Essentially all thymocytes and most spleen and bone marrow cells expressed T200 glycoprotein. About 10% of spleen and bone marrow cells expressed less T200 glycoprotein than that required to distinguish specific fluorescence from nonspecific staining. The distribution of T200 glycoprotein on spleen cells was more heterogenous than on thymocytes but both cell types expressed very similar amounts of antigen. The distribution of T200 glycoprotein on bone marrow cells was bimodal with the two cell populations, present in about equal numbers, expressing 30–70% of the amount of T200 glycoprotein found on thymocytes. In comparison only 23% of spleen cells and less than 2% of bone marrow cells were positive for Thy-1 glycoprotein.

Cytotoxicity of Anti-T200 Antibody in the Presence of Absorbed Rabbit Complement

One explanation for the selective cytotoxicity of anti-T200 antibody for mature T cells was that the rabbit serum used as a source of complement contained sublytic amounts of contaminating antibodies against surface antigens present on these cells but not on their progenitors nor mature hematopoietic cells of other lineages. This was investigated by absorbing the complement three times with 1×10^8 R1(TL^+) thymoma cells/milliliter. Before absorption, the rabbit serum killed 100% of R1(TL^+) cells at a dilution of 1:3 and 50% R1(TL^+) cells were killed at a dilution of 1:10. About 20% of thymocytes were killed by the unabsorbed antibody at a dilution of 1:3. After absorption, less than 10% R1(TL^+) cells were killed by the rabbit serum at 1:3, and no killing of thymocytes was observed at this dilution. Using the absorbed rabbit serum as a source of complement at a dilution of 1:12 for R1(TL^+) cells and 1:5 for thymocytes, anti-T200 antibodies still completely killed both cells to a dilution of 1:160 with 50% lysis at between 1:320 to 1:640 (compare with Fig. 1). It is clear therefore that contaminating antibodies in the rabbit serum used as the complement source do not play a role in the selective killing of T cells by anti-T200 monoclonal antibody.

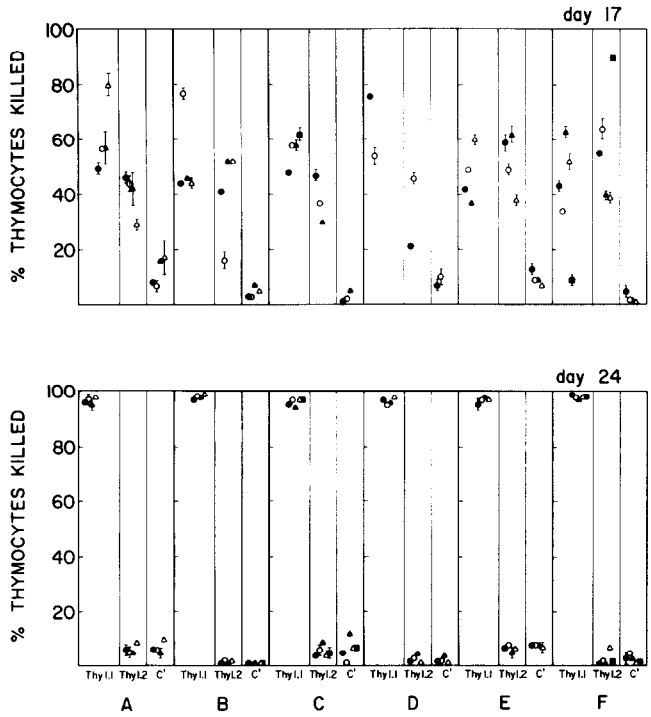

FIG. 2. Effects of anti-T200 and anti-Thy-1 antibodies on prothymocytes. AKR/J bone marrow cells were treated with antibody and complement as described under Materials and Methods and tested for their capacity to repopulate thymuses of irradiated AKR/Cum mice. The figure shows the percentage of Thy 1.1 and Thy 1.2 cells in the thymuses of individual recipient mice 17 or 24 days after injection with bone marrow cells treated as follows: Group A, untreated; Group B, C' alone; Group C, anti-T200 + C'; Group D, anti-T200 alone; Group E, anti-Thy-1 + C'; Group F, anti-Thy-1 alone.

DISCUSSION

The results reported here clearly demonstrate that a monoclonal antibody against T200 glycoprotein is selectively cytotoxic for T lymphocytes and that sensitivity to

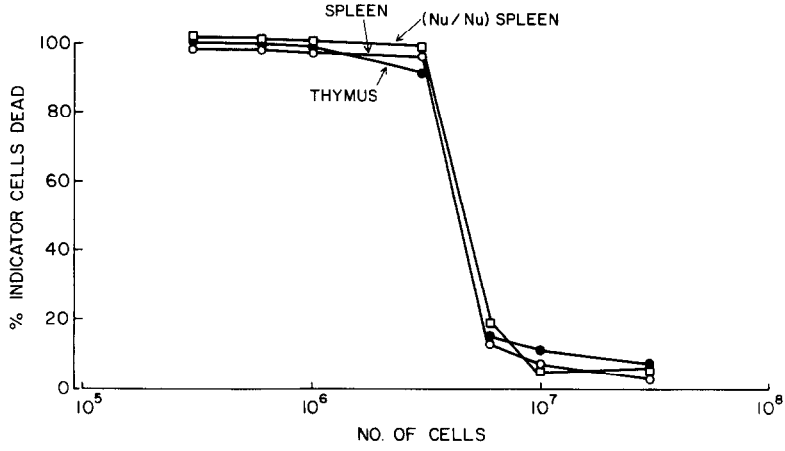

FIG. 3. Relative amount of T200 glycoprotein on BALB/c spleen and thymus and BALB/c (Nu/Nu) spleen cells estimated by quantitative cytotoxic absorption. See Materials and Methods for experimental details.

lysis is acquired shortly before or just after migration of thymic precursor cells into the thymus. This selective cytotoxicity which can be observed over a wide range of experimental conditions does not appear to be a property of the particular monoclonal antibody we have studied in detail but rather is a characteristic of T200

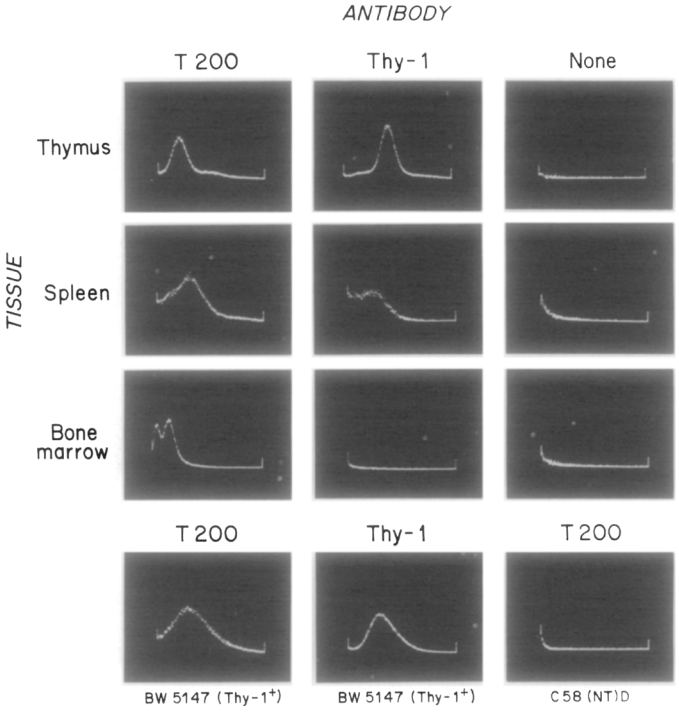

FIG. 4. Quantitative expression of T200 glycoprotein on thymus, spleen, and bone marrow cells determined by fluorescence-activated cell analysis. Cell suspensions were prepared and stained with FITC-labeled antibody as described under Materials and Methods. Cells were analyzed on the fluorescence-activated cell sorter using a laser output of 400 mW and high-voltage output of 900 V. Cell number was estimated by light scatter and 200,000 cells were analyzed. Fluorescent beads of known diameter were used as size standards, 5-μm beads were at channel 230, and 10-μm beads at channel 770 of the 1024 channel display. Fluorescent gain and display were varied depending on the fluorescence intensity of the labeled cells. The same gain was used for analysis of each antibody on all normal lymphoid cell populations. Abscissae represents relative fluorescence intensity; ordinates give relative cell number. The percentage of fluorescent cells was determined by integration between the channels contained in the region between the cursors shown in each display. These values and the relative intensity of fluorescence calculated from the position of the fluorescence peaks for each cell suspension and antibody are given below. Less than 2% of cells were fluorescent when the first stage antibody was omitted. C58(NT)D is a rat lymphoma cell line used as a negative control.

	Antibody			
	T200		Thy-1	
Cells	% Labeled cells	Relative peak intensity	% Labeled cells	Relative peak intensity
Thymus	91	1.0	94	5.9
Spleen	78	1.4	23	4.1
Bone marrow	81	0.35, 0.74	1.7	—
BW5147(Thy-1+)	90	3.5	99	10.4

glycoprotein itself. Sensitivity to complement-mediated lysis by anti-Thy-1 and anti-T200 antibodies appears to develop at a similar stage of T-cell differentiation. In the case of Thy-1, this can be accounted for by the increased amount of the antigen found on the surface of thymocytes. During fetal development, it has been shown that the thymus is initially populated by immigrant cells which, by indirect immunofluorescence, are Thy-1 negative, but subsequently give rise to Thy-1-positive cells (12). It is clear from the fluorescence-activated cell analysis of the expression of T200 glycoprotein on thymus, spleen and bone marrow cells that the amount of T200 glycoprotein found on the cell surface cannot be the only factor that determines sensitivity to lysis by anti-T200 antibodies even though in some situations small changes in antigen expression can have marked effects on complement-mediated lysis (13). Two other explanations may be considered. First, it is known that there are structural differences between the glycoproteins recognized by anti-T200 antibodies on different hematopoietic cells which can be detected by SDS–polyacrylamide gel electrophoresis (1, 3). These differences may influence the efficiency of complement-mediated lysis. Second, it is also possible that the organization of T200 glycoprotein molecules in the plasma membrane of thymocytes and mature T cells differs from other cell types leading to differences in the binding of complement components. Clearly, therefore, although pluripotent stem cells and prothymocytes are not killed by anti-T200 antibodies, this does not imply that these cells express less T200 glycoprotein than thymocytes and mature T cells. In principle, information about the quantitative distribution of antigens on functional cell types may be obtained by fluorescence-activated cell sorting in conjunction with the appropriate functional assay. It should be noted, however, that sorting sufficient numbers of cells to carry out certain functional assays, for example, the prothymocyte assay, may present practical problems. As judged by functional assays, the distinction between T and B lymphocytes in terms of their sensitivity to lysis by anti-T200 antibody is clearcut. However, anti-T200 antibody consistently kills somewhat higher numbers of bone marrow and spleen cells than each of the three anti-Thy-1 monoclonal antibodies we have tested. The reason for this is not known although one possibility is that another hematopoietic cell population in addition to T cells is sensitive to lysis by anti-T200 antibody. However, there is no direct evidence to support this notion. There is little effect of pretreating spleen cells with anti-T200 antibody and complement on either the effector step of antibody-dependent cell-mediated cytotoxicity or on natural-killer cell activity (G. Dennert, unpublished results). Finally, the results reported here emphasize the limitations of complement-mediated cytotoxicity as a method for assessing the distribution of cell-surface antigens on different cell types. Thus, suggestions that monoclonal antibodies recognize cell-surface antigens specific for subpopulations of lymphocytes based on direct cytotoxicity studies should be assessed cautiously. On the other hand, the possibility that antibodies recognizing antigens widely distributed on different cell types may nevertheless display a much more restricted pattern of cytolytic activity has important practical implications, for example, anti-T200 antibody could be used in the same manner as anti-Thy-1 antibody to eliminate T cells from heterogenous cell populations.

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