

## THE SUBDIVISION OF THE T4 (CD4) SUBSET ON THE BASIS OF THE DIFFERENTIAL EXPRESSION OF L-C/T200 ANTIGENS

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mAbs have been used successfully to identify antigens on the surface of T cells such as T4 (CD4) and T8 (CD8), which can subdivide lymphocytes into discrete subsets. The T4 antigen at 67,000  $M_r$  denotes a subset with helper activity and is involved in the recognition of HLA-D region antigens (1, 2). The T8 antigen at 32,000  $M_r$ /34,000  $M_r$  appears to identify a subset with suppressor/cytotoxic activity and recognizes MHC class I antigens (3). Recently, it has become evident that the T4<sup>+</sup> subset of T cells is a heterogeneous collection of cells with different phenotype and function. In particular, two mAbs designated anti-2H4 and anti-4B4 have been found to distinguish reciprocal subsets of T4<sup>+</sup> cells with suppressor inducer activity (T4<sup>+</sup>,2H4<sup>+</sup>) and helper activity (T4<sup>+</sup>,4B4<sup>+</sup>) for B cells (4, 5). The T4<sup>+</sup>,2H4<sup>+</sup> subset, while not directly suppressive, can induce cells within the T8<sup>+</sup> subset to suppress Ig production by B cells in response to PWM and specific antigen (5, 6). In contrast, the T4<sup>+</sup>,4B4<sup>+</sup> subset can provide help to B cells, and has been defined by a number of mAbs such as UCHL1 (7) and WR19 (8).

These distinct subsets within the T4(CD4) population also appear to be differentially triggered by various stimuli to elicit their immune functions. The T4<sup>+</sup>,2H4<sup>+</sup> subset appears to be preferentially responsive to self-Ia in the autologous MLR (AMLR)<sup>1</sup> (9). In contrast, the response of T4<sup>+</sup> cells to soluble antigen is virtually restricted to the T4<sup>+</sup>,4B4<sup>+</sup> subset. Both subsets of cells respond strongly to alloantigen in the MLR (6). After activation, the expression of the 2H4 antigen on the surface of cells increases, which in turn is correlated to the level of suppressor function induced by the T4<sup>+</sup>,2H4<sup>+</sup> subset (6, 9). The mAb antibody to the 2H4 antigen on the activated T4<sup>+</sup>,2H4<sup>+</sup> cells is also capable of abrogating the ability of these cells to induce suppression (6, 9). Although the molecular basis of this blockage is unclear, these data have suggested a role for the 2H4 antigen in the regulation of the immune response.

The leucocyte common antigen (L-C)/T200 family of antigens of  $180 \times 10^3$  to  $\sim 240 \times 10^3 M_r$  are well established as a major group of antigens on the surface

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<sup>1</sup> Abbreviations used in this paper: AMLR, autologous MLR; L-C, leucocyte common antigen.

of lymphocytes (10, 11). The cDNA sequence of the L-C/T200 antigen encodes for a highly glycosylated polypeptide of which at least 700 amino acids make up the cytoplasmic tail (12, 13). Intriguingly, individual forms of the L-C antigens appear to be expressed differentially on subsets of lymphoid cells (14–20). Thymocytes have been reported to express a single isoform at  $180 \times 10^3$ , while T lymphocytes express multiple forms at  $180$ – $220 \times 10^3$  and B cells express a single form at  $240 \times 10^3$  (15–17, 20). Each isoform is probably generated by differences in the extracellular portion of the polypeptide (21–23). These variants have in turn been found to express novel epitopes that can be used to distinguish B cells (15), T cells (24), Th cells (20) and cytotoxic T lymphocytes (17, 18) within the lymphoid population. This heterogeneity in the expression of L-C/T200 antigens on the surface of different lymphoid cells is consistent with a role of the individual isoforms in the differentiation and function of the immune system. The L-C/T200 antigens have been implicated in B cell differentiation (25) and NK cytotoxicity (26); however, additional information is needed to clarify the role of these structures in immunity.

Given the importance of subdividing the T4 (CD4) cells into distinct functional subsets, we attempted to understand the distinction in molecular terms between the suppressor/inducer ( $T4^+, 2H4^+$ ) and helper ( $T4^+, 2H4^-$ ) cells. The 2H4 antigen was found to comprise three distinct polypeptides (125, 200, and  $220 \times 10^3$ ), of which two forms at 200 and  $220 \times 10^3 M_r$  are members of the L-C/T200 family of antigens. Importantly, the distinction of the T cell compartment into the  $T4^+, 2H4^+$  and  $T4^+, 2H4^-$  subsets is characterized by the differential expression of the 200 and  $220 \times 10^3 M_r$  isoforms of the L-C/T200 antigen. Biosynthetic studies have shown that the 2H4 subunits at 200 and  $220 \times 10^3 M_r$  are derived from distinct polypeptides at 150 and  $160 \times 10^3$  by the addition of O-linked and N-linked oligosaccharides. The third 2H4 subunit at  $125 \times 10^3$  is nonglycosylated and appears resistant to iodination at the cell surface. An antibody specific for the 200/ $220 \times 10^3 M_r$  isoforms was capable of abrogating the function of the  $2H4^+$  subset of  $T4(CD4)^+$  cells.

### Materials and Methods

*Monoclonal Antibodies.* The production and characterization of the anti-2H4 (IgG1) and the anti-4B4 (IgG1) antibodies has been described elsewhere (4, 5). The anti-2H4 antibody reacted with 40–50% of T4 cells and some 60% of T8 lymphocytes. The mAbs 9.4 (IgG2) (a kind gift from Dr. P. Martin, Seattle, WA), PD7/26, and 2B-11 (IgG1) (Dako Corp., Santa Barbara, CA) have been previously reported to react with L-C/T200 antigens (27, 28).

*Purification of Various Lymphoid Populations.* Human PBL were isolated from healthy volunteer donors by Ficoll-Hypaque density gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ). Unfractionated lymphocytes were first depleted of macrophages by adherence to plastic as previously described (4). The macrophage-depleted lymphocytes were then separated into erythrocyte rosette (E-rosette) positive ( $E^+$ ) and E rosette-negative ( $E^-$ ) populations with 5% sheep erythrocytes (Microbiological Associates, Walkersville, MD). The T cell population thus obtained was 95%  $E^+$  and 94% reactive with an anti-T3 mAb. This T-enriched population was then cultured with Con A (5  $\mu\text{g/ml}$ ) in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 1% (vol/vol) penicillin-streptomycin for 48 h before radiolabeling.

The  $T4^+$  cells were separated into  $T4^+, 2H4^+$  and  $T4^+, 2H4^-$  subpopulations by anti-Ig-coated plates as described (4). In brief,  $12 \times 10^6$   $T4^+$  cells were exposed to 1 ml of anti-

2H4 antibody (a 1:125 dilution of ascites) for 30 min at 4°C and washed to remove excess antibody. Some  $12 \times 10^6$  cells were then suspended in 3 ml medium and applied to a goat anti-mouse Ig antibody-coated plastic plate (Fisher Scientific Co., Pittsburgh, PA). After 70 min of incubation at 4°C, nonadherent and adherent populations were collected. The adherent population ( $T4^+, 2H4^+$ ) was found to be consistently 95% positive for the 2H4 antigen as assessed by antibody binding using an Epics cell sorter. In contrast, the nonadherent population ( $T4^+, 2H4^-$ ) was only marginally positive (4%), as assessed by staining with the anti-2H4 antibody.

*Assay for Suppressor/Inducer Activity in PWM-stimulated Ig Synthesis.* AMLR cultures were prepared in RPMI 1640 supplemented with 10% human AB serum, 200 mM L-glutamine, 25 mM Hepes buffer (Microbiological Associates), 0.5% sodium bicarbonate, and 1% penicillin-streptomycin. For a primary culture of AMLR,  $10^7$  responder T cells were cocultured with  $10^7$  irradiated (5,000 rad) autologous E cells in a total volume of 10 ml of medium in 25-cm<sup>2</sup> culture flasks (Falcon Labware, Oxnard, CA). After 7 d, culture cells were layered over Ficoll-Hypaque and centrifuged at 2,000 rpm for 20 min, and then washed three times. AMLR-activated T cells thus obtained were added to secondary cultures to determine the immunoregulatory functions of these cells.

Unfractionated T cells ( $10^6$ /ml) were cultured with Con A (Sigma Chemical Co., St. Louis, MO) in the presence of 5% macrophages in RPMI 1640 containing 20% FCS, 1% penicillin-streptomycin, 200 mM L-glutamine, 25 mM Hepes buffer, and 0.5% sodium bicarbonate at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were cultured for 2 d, harvested, and treated with 0.1 M  $\alpha$ -methyl-D-mannoside for 30 min before the addition of cells to the secondary culture. To remove dead cells, cultured cells were layered over Ficoll-Hypaque and centrifuged at 2,000 rpm for 20 min and then washed four times.

AMLR-activated T4 cells ( $10^6$ ) or Con A-activated T cells were treated with 1 ml of monoclonal anti-2H4, 9.4, PD721, and 2B-11 antibodies (1:100 dilution) for 60 min at 4°C without complement. The activated cells that had been treated with antibody were then washed extensively and added to a secondary culture for the assessment of the suppression of PWM-driven IgG synthesis. Either the AMLR-activated T4 cells or the Con A-activated T cells were then added to fresh autologous responding lymphocytes at the initiation of MLR and PWM-stimulated IgG synthesis cultures. Suppression of a PWM-driven IgG synthesis was assessed in round-bottomed microtiter plates with wells containing PWM and  $10^5$  autologous unfractionated lymphocytes and varying numbers of control lymphocytes or AMLR/Con A-activated T cells. After 7 d, cultures were terminated, supernatants were harvested, and IgG secretion was assayed by RIA.

*Radio-labeling.* Intact cells (95% viable) were labeled by lactoperoxidase-catalyzed iodination as previously described (29).

The biosynthetic labeling of cells with [<sup>35</sup>S]methionine has been described previously (30). Briefly, cells at  $10^8$ /ml were labeled with 1.0 mCi of [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine (New England Nuclear, Boston, MA) in cysteine-, methionine-free RPMI 1640 medium supplemented with 1% (vol/vol) dialyzed FCS for 4 h at 37°C. After washing twice with RPMI 1640 medium,  $2 \times 10^7$  cells were lysed in 1 ml of lysis buffer (10 mM Tris/HCL buffer, pH 8.2, containing 1% (vol/vol) NP-40, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF). The lysates were left on ice for one-half hour before centrifugation in a centrifuge (13,000 g; Eppendorf; Fisher Scientific Co.) for 10 min. Cell lysates were either used immediately or stored at -70°C.

For tunicamycin treatment, cells ( $5 \times 10^6$  cells/ml) were preincubated for 1 h in methionine, cysteine-free RPMI 1640 medium supplemented with 20  $\mu$ g/ml of tunicamycin (Sigma Chemical Co.). Cells were then centrifuged and resuspended at  $10^8$  cells/ml with [<sup>35</sup>S]cysteine, methionine, and tunicamycin for pulse-chase analysis. Pulse-chase experiments were performed by incubating cells ( $10^8$ /ml) for 10 min with [<sup>35</sup>S]methionine (1 mCi/ml) and [<sup>35</sup>S]cysteine (1 mCi/ml), before washing three times with RPMI 1640 medium and chasing at  $5 \times 10^6$  cells/ml for the indicated times in RPMI 1640 medium containing 2.5 mM cold cysteine, methionine.

*Immunoprecipitation Analysis.* Cell lysates (1 ml) were precleared overnight at 4°C with

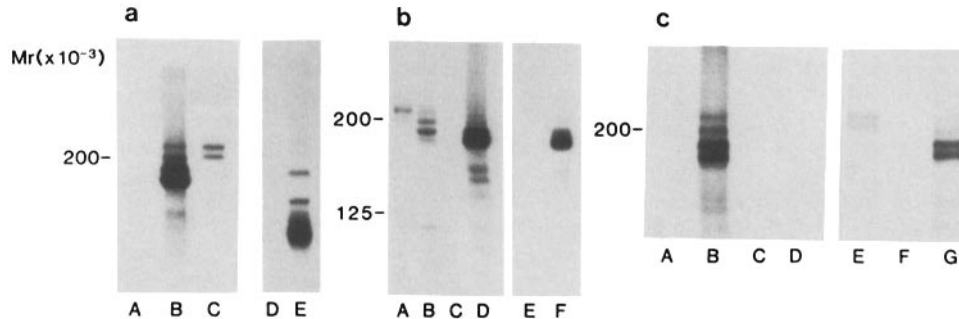
about 100  $\mu$ l of 10% (wt/vol) of formaldehyde-fixed *Staphylococcus aureus* Cowan Strain 1 (SAC1) bacteria. The precleared lysate was then incubated at 4°C for either 1–2 h or overnight with soluble mAb (10–20  $\mu$ g of antibody), or with 50  $\mu$ l of a 10% (wt/vol) mixture of mAb coupled to protein A–Sepharose beads. The antibodies were coupled to beads at a concentration of 10 mg/ml by use of the crosslinker dimethylpimelidate-HCl (30 mM) as previously described (31). In the case of soluble antibody, some 50  $\mu$ l of 10% (wt/vol) protein A–Sepharose was incubated with the lysate for 1–2 h at 4°C or overnight before precipitation. Precipitates were washed once in lysis buffer containing 0.1% (wt/vol) SDS and twice in lysis buffer alone. Beads were then eluted for enzymatic digestion, or boiled for 4 min in 50  $\mu$ l of 0.0625 M Tris-HCl, pH 6.8, with 2.0% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue. Samples were then run on SDS-PAGE as described by Laemmli (32). A gradient gel of 5–7.5% was found optimal for the separation of polypeptides in the 200–125  $\times 10^3 M_r$  range. Gels were then fixed and subjected to autoradiography or fluorography by use of  $^3\text{H}$ -Enhance (New England Nuclear) in conjunction with light-intensifying screens (Cronex Lighting Plus; Dupont Co., Wilmington, DE) and Kodak X-R5 film (Eastman Kodak Co., Rochester, NY) at  $-70^\circ\text{C}$ .

**Enzymatic Digestions.** Glycosidase digestions were carried out by elution of immune complexes in 30  $\mu$ l of 0.5% (wt/vol) SDS from protein A–Sepharose by boiling for 4 min and the subsequent addition of 120 ml of the enzyme reaction mixture. Neuraminidase digestion was carried out by adding 120  $\mu$ l of a sodium citrate buffer, pH 5.5, containing 0.3 M NaCl and 0.2%  $\text{CaCl}_2$  in conjunction with 100 mU of neuraminidase (Sigma Chemical Co.). For treatment with endo H, 1–2 U of endo H (New England Nuclear or Genzyme, Boston, MA) was added to 120  $\mu$ l of 150 mM sodium citrate, pH 5.5. Endo F treatment was done by the addition of 120 ml of 150 mM sodium phosphate, pH 6.1, containing 50 mM EDTA, 1% (vol/vol) NP-40, 1% (vol/vol) 2-ME, and 1–2 mU of enzyme. Treatment with *O*-glycanase (Genzyme) was conducted by the addition of 120  $\mu$ l of a 20 mM Tris-maleate buffer, pH 6.0, containing 10 mM calcium acetate, 10 mM D-galactono- $\beta$ -lactose, and 100 mU of neuraminidase. This mixture was allowed to incubate at 37°C for one-half hour before the addition of 1–2 mU of *O*-glycanase for 12 h at room temperature. Neuraminidase-treated controls were used as an internal control to determine whether the shift in  $M_r$  was due to the removal of sialic acid and/or *O*-linked oligosaccharides.

The protease inhibitor PMSF in acetone was added to each of the above mixtures to a final concentration of 1 mM. After digestion, reaction mixtures were precipitated in siliconized microfuge tubes by the addition of 30  $\mu$ g of BSA and an equal volume of 30% (wt/vol) of TCA for one-half hour on ice followed by three washes with acetone at  $-20^\circ\text{C}$ . Occasionally, the reaction mixtures were precipitated with 95% (vol/vol) ethanol for a period of 2 d at  $-20^\circ\text{C}$ . The precipitates were then dried in a desiccator and stored at room temperature.

## Results

**Structural Comparison of the 2H4 and 4B4 Antigens.** A structural comparison of the 2H4 and 4B4 antigens was initially conducted to determine whether the subdivision of the T4 (CD4) peripheral T cells into distinct subsets was mediated by similar or distinct polypeptides. Fig. 1a reveals the pattern of polypeptides recognized by the antibodies from T-enriched cultures of Con A–activated cells that had been labeled by lactoperoxidase-catalyzed iodination. Under these conditions, the anti-2H4 antibody precipitated two subunits at 200 and 220  $\times 10^3 M_r$  of equal approximate intensity (lane C). In contrast, the anti-4B4 antibody precipitated several different polypeptides at 135, 165, and 185  $\times 10^3 M_r$  (lane E). The intensity of 135  $\times 10^3 M_r$  chain greatly exceeded that of the 165 and 185  $\times 10^3 M_r$  bands, which were of about the same intensity. Occasionally, several other faint bands at 80 and 210  $\times 10^3 M_r$  were also visualized. Thus, the



**FIGURE 1.** (a) The structure of the 2H4, 4B4, and L-C/T200 antigens expressed on the surface of T cells. Con A-stimulated peripheral blood E rosette-positive cells (>95%) were labeled by lactoperoxidase-catalyzed iodination and lysed in NP-40 lysis buffer for immunoprecipitation as previously described (30). (A) Rabbit anti-mouse control; (B) the 9.4 antibody; (C) anti-2H4 antibody; (D) rabbit anti-mouse control; (E) anti-4B4 antibody. (b) The structure of the 2H4 and L-C/T200 antigens expressed on  $T4^+, 2H4^+$  cells and  $T4^+, 2H4^-$  cells. Peripheral blood E rosette-positive cells were fractionated by antibody plating (see Materials and Methods) into  $T4^+, 2H4^+$  (A and B) and  $T4^+, 2H4^-$  (C and D) subsets. These T cells and  $T4^+, 2H4^-$  thymocytes (E and F) were labeled by lactoperoxidase-catalyzed iodination (29). (A, C, and E) Anti-2H4 antibody. (B, D, and F) 9.4 antibody. (c) Sequential immunodepletion of the 2H4 and L-C/T200 antigens. Lysates were precleared with either the 9.4 antibody (B and C) or the anti-2H4 antibody (E and F) before precipitation with the anti-2H4 antibody (D) or the 9.4 antibody (G). (A) Rabbit anti-mouse control; (B) 9.4 antibody (1st precipitation); (C) 9.4 antibody (5th precipitation); (D) anti-2H4 antibody. (E) Anti-2H4 antibody (1st precipitation); (F) anti-2H4 antibody (5th precipitation); (G) 9.4 antibody.

2H4 and 4B4 antigens appear to constitute a distinct spectrum of polypeptides as expressed on the different subsets of T4 cells.

Given the observation that the anti-2H4 antibody precipitated several polypeptides in the  $M_r$  range of  $200 \times 10^3$ , it was next of interest to determine whether these polypeptides were related to any of the L-C antigens. The L-C antigens, termed T200 antigens on T cells, make up a major group on Con A-binding proteins in the  $200 \times 10^3 M_r$  range on the surface of haematopoietic cells (10, 34). The 9.4 antibody has previously been shown to be reactive with a spectrum of L-C/T200 antigens (27). Fig. 1a reveals the pattern precipitated by the 9.4 antibody of bands at 180, 190, 200, and  $220 \times 10^3 M_r$  (lane B). The subunits at 200 and  $220 \times 10^3$  precipitated by the anti-2H4 antibody appeared to comigrate with the high  $M_r$  versions of the L-C/T200 antigens (lanes B and C). In contrast, the 185 form of the 4B4 antigen appeared to be located midway between the L-C/T200 subunits at 180 and  $190 \times 10^3 M_r$  (lane E). The similarity in molecular size of the 2H4 antigens with the higher  $M_r$  versions of the L-C/T200 antigens introduced the possibility that these antigens may be structurally related.

*The Expression of the 2H4 and L-C/T200 Antigens on the  $T4^+, 2H4^+$  and  $T4^+, 2H4^-$  Subsets.* The nature of the 2H4 and L-C/T200 antigens on the surface of the  $T4^+, 2H4^+$  helper subset was then contrasted with the expression of these polypeptides on the  $T4^+, 2H4^-$  suppressor/inducer subset of peripheral blood cells. As shown in Fig. 1b, the anti-2H4 antibody precipitated two polypeptides at  $200 \times 10^3$  and  $220 \times 10^3$  from  $T4^+, 2H4^+$  cells (lane A), while the 9.4 antibody reacted with a spectrum of polypeptides at 180, 190, 200, and  $220 \times 10^3 M_r$  (lane B). The intensity of the chains at 190 and  $200 \times 10^3 M_r$  tended to be

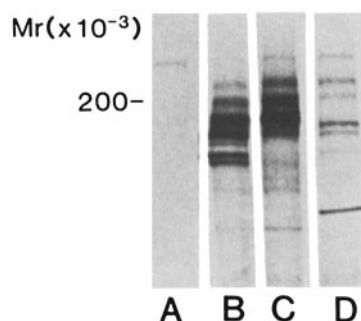


FIGURE 2. The structure of the 2H4 and L-C/T200 antigens as assessed by biosynthetic labeling. Con A-stimulated T cells were cultured for 2 d and subsequently labeled with [ $^{35}$ S]cysteine, methionine for 4 h before immunoprecipitation analysis. (A) Rabbit anti-mouse control. (B) 9.4 antibody. (C) PD7/26 and 2B-11 antibodies. (D) Anti-2H4 antibody.

greater than the 180 and  $220 \times 10^3$  bands on the  $T4^+, 2H4^+$  subset. In contrast, no material was detected from the  $T4^+, 2H4^-$  cells when the anti-2H4 antibody was used to precipitate antigen (lane C). However, from the same cells, the 9.4 antibody precipitated two major polypeptides at 180 and  $190 \times 10^3 M_r$  (lane D). Occasionally, several less prominent bands at  $\sim 160$  and  $170 \times 10^3$  were also observed. A similar correlation between the absence of anti-2H4 reactivity and the absence of the 200 and  $220 \times 10^3 M_r$  polypeptides were observed for thymocytes of the  $T4^+, 2H4^-$  phenotype. In this case, the thymocytes expressed both the 180 and  $190 \times 10^3$  bands without material at a higher  $M_r$  (lane F). No bands were detected in immunoprecipitates formed by the anti-2H4 antibody (lane E). Interestingly, the absence of the higher  $M_r$  polypeptides appeared to be accompanied by an increase in the level of expression of the lower  $M_r$  species at 180 and  $190 \times 10^3$ . Each of these precipitations was performed at the same time with the same number of cells and radioactivity. This observation indicated that the functional subdivision of T4 cells into helper cells and suppressor/inducer cells appeared to be accompanied by the differential expression of the T200 antigens. Furthermore, the absence of the high  $M_r$  forms of the L-C/T200 antigens with the reactivity of the anti-2H4 antibody implied that the 2H4 antigen may be a member of the L-C/T200 family of antigens.

*The 2H4 Antigen Is a Member of the L-C/T200 Family of Polypeptides.* To assess directly whether the 2H4 antigen(s) were members of the L-CA family, the anti-2H4, and anti-L-C/T200 antibodies were used in sequential immunodepletion analysis. Fig. 1c reveals the pattern in which the 9.4 antibody was used to deplete antigen (lanes B and C) from a lysate that was subsequently challenged with the anti-2H4 antibody (lane D). The 9.4 antibody completely depleted antigen that was reactive with the anti-2H4 antibody (lanes B-D). In contrast, depletion of the lysate with the anti-2H4 antibody resulted in the complete removal of the subunit at  $220 \times 10^3 M_r$  and caused a reduction in the intensity of the subunit at  $200 \times 10^3 M_r$  (lanes E-G). These data show clearly that the 2H4 antigen(s) constitute a subset of antigens within the L-C/T200 family.

*The 2H4 Antigen Comprises a Novel Antigen as Assessed by Biosynthetic Labeling.* An important question was to determine whether the anti-2H4 antibody reacted with any polypeptides distinct from those recognized by antibodies that were broadly reactive with the L-C/T200 antigens. Fig. 2 shows the SDS-PAGE pattern of polypeptides derived from peripheral T cells that had been stimulated with Con A and labeled for 6 h with [ $^{35}$ S]cysteine, methionine. Under these

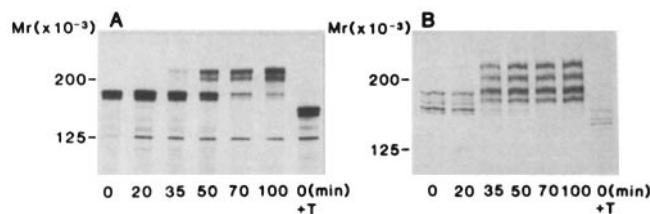


FIGURE 3. (A) Pulse-chase analysis of the 2H4 antigen. Cultures of T-enriched peripheral blood lymphocytes were stimulated from 2 d with Con A and then labeled for 10 min with [ $^{35}$ S]cysteine, methionine (chase period 0). Cells were then chased in the presence of complete media for

20, 35, 50, 70, and 100 min before solubilization and immunoprecipitation using the anti-2H4 antibody. Tunicamycin treatment involved a preincubation in the presence of the antibiotic (20  $\mu$ g/ml) before the addition of radioisotope for 10 min. (B) Pulse-chase analysis of the L-C/T200 antigens. Cultures of T-enriched PBL were stimulated with Con A and subjected to pulse-chase analysis as described above in A.

conditions, the anti-2H4 antibody was found to precipitate a series of subunits at 125, 150, 160, 175, 185, 200, and  $220 \times 10^3 M_r$  (Fig. 2D). The 200 and  $220 \times 10^3 M_r$  subunits appeared to correspond to the 2H4 subunits precipitated from the cell surface (Fig. 1a, lane C). In contrast, the anti-L-C/T200 antibodies 9.4, PD7/26, and 2B-11 precipitated a more complex collection of polypeptides ranging from  $110$ – $220 \times 10^3$ . Of these, the 9.4 antibody recognized a number of major bands at  $140$ – $220 \times 10^3$  (Fig. 2B), while the combination of PD7/26 and 2B-11 antibodies reacted primarily with several subunits at  $175$ – $220 \times 10^3$  (Fig. 2C). These antibodies have previously been found to react with a broad range of T200 antigens (27, 28). The 2H4 subunits at  $150$ – $220 \times 10^3$  could be accounted for within the series of bands precipitated by the anti-L-C/T200 antibodies. However, the anti-2H4 antibody also appeared to precipitate a prominent polypeptide at  $125 \times 10^3 M_r$  that was not detectable in the anti-L-C/T200 immunoprecipitate (Fig. 2, lane D, lowest arrow). This subunit was well represented in the precipitations relative to the other 2H4 subunits from biosynthetically labeled cells, but was not detected in precipitates from iodinated cells (Fig. 1a, lane C). Thus, the anti-2H4 antibody reacts with a unique subunit at  $125 \times 10^3$  in addition to a subset of the L-C/T200 family of antigens.

**Biosynthetic Interrelationships of the Individual 2H4 Subunits.** It was next important to establish whether each of the 2H4 subunits in the  $125$ – $220 \times 10^3 M_r$  range were biosynthetically interrelated or constituted distinct polypeptides. Previous studies (18) have shown that individual polypeptides within the L-C/T200 family can undergo changes in molecular size due to differences in glycosylation. Fig. 3A reveals the pattern of 2H4 subunits from a pulse-chase experiment in which Con A-stimulated cells were labeled for 10 min with radioactive cysteine and methionine, and chased with unlabeled cysteine and methionine for various intervals up to 100 min. After a 10-min labeling period (chase period 0), the anti-2H4 antibody precipitated two major bands at  $175$  and  $185 \times 10^3$  and a faint band at  $125 \times 10^3$ . At this stage, glycosylation is thought to be restricted to the addition of N-linked oligosaccharides (35). In contrast, when cells were labeled in the presence of tunicamycin, the anti-2H4 antibody precipitated two bands at  $150$  and  $160 \times 10^3$  as well as a band at  $125 \times 10^3$  (Fig. 3A, last lane). Tunicamycin is an antibiotic that inhibits N-linked glycosylation of polypeptides (36). These data suggest that the 2H4 doublet of  $175/185 \times 10^3 M_r$  is derived from two distinct polypeptides at  $150$  and  $160 \times 10^3 M_r$  by the

addition of multiple *N*-linked oligosaccharides. A difference in  $M_r$  of  $25\text{--}30 \times 10^3$  is consistent with the addition of some 12–14 *N*-linked oligosaccharides. Lastly, the fact that the anti-2H4 antibody reacted with the nonglycosylated versions of the antigen indicates that the antibody recognizes the protein backbone of the 2H4 antigen.

As the chase period was extended to 20–35 min, the 2H4 antibody began to precipitate a new set of polypeptides at  $200$  and  $220 \times 10^3$ . At this time, the *N*-glycosylated proteins have reached the Golgi apparatus where the maturation of *N*-linked glycans and the addition of *O*-linked oligosaccharides can occur (37). As the chase time was increased, the intensity of the bands at  $200$  and  $220 \times 10^3 M_r$  increased with a concomitant decrease in the intensity of the  $175$  and  $185 \times 10^3 M_r$  subunits. This change in the relative intensities of bands suggests that the  $175/185 \times 10^3 M_r$  subunits are processed to a form at  $200/220 \times 10^3 M_r$ , a form detected at the surface of T cells (Fig. 1*a*, lane C). In contrast, the intensity of the  $125 \times 10^3 M_r$  increased during the initial chase period of 0–20 min, after which it remained at the same approximate intensity throughout the chase period (Fig. 3*A*). The absence of any detectable change in  $M_r$  suggested that this subunit is nonglycosylated and biosynthetically unrelated to the other 2H4 antigens.

By contrast, the 9.4 antibody precipitated at least five distinct isoforms of the L-C/T200 antigen from cells that had been labeled with [ $^{35}\text{S}$ ]cysteine, methionine in a pulse-chase experiment. As shown in Fig. 3*B*, after a 10-min period of labeling, the 9.4 antibody was found to precipitate five bands at  $\sim 155$ ,  $160$ ,  $170$ ,  $175$ , and  $185 \times 10^3 M_r$ . Given the short period of labeling, these versions most likely correspond to the L-C/T200 antigens after the addition of *N*-linked oligosaccharides. In contrast, the same antibody precipitated five bands at  $\sim 130$ ,  $135$ ,  $145$ ,  $150$ , and  $160 \times 10^3 M_r$  from cells that had been labeled for the same period in the presence of tunicamycin (Fig. 3*B*, last lane). Importantly, this pattern indicates the existence within the cell of at least five nascent polypeptides corresponding to different isoforms of the T200 antigen. An  $M_r$  difference of some  $20\text{--}25 \times 10^3$  was observed between the glycosylated and nonglycosylated forms of the T200 antigens, as observed for the 2H4 isoforms of these antigens (Fig. 3*A*). Lastly, under both conditions of labeling, the anti-2H4 antibody recognized the two highest  $M_r$  isoforms of the L-C/T200 antigens precipitated by the 9.4 antibody.

Over a chase period of 20–35 min, the 9.4 antibody began to precipitate additional material at  $180$ ,  $190$ ,  $200$ , and  $220 \times 10^3 M_r$  (Fig. 3*B*). These forms of the antigen appear to correspond to the versions derived from the surface of iodinated cells (Fig. 1*A*, lane C). As observed in the case of the 2H4 antigen, when the period of chase was extended, the intensity of the lower  $M_r$  isoforms decreased with a concomitant increase in the intensity of the high  $M_r$  forms. Interestingly, the five distinct isoforms of the L-C/T200 bands gave rise to only four bands as assessed by SDS-PAGE analysis. It is therefore possible that one or more of the  $180$ ,  $190$ ,  $200$ , and  $220 \times 10^3 M_r$  bands from the surface of cells comprises multiple isoforms of the L-C/T200 antigens.

*Endoglycosidase Digestion of the 2H4 Subunit.* In an attempt to directly ascertain whether the above assignments were correct, immunoprecipitates formed by the anti-2H4 antibody were digested with various glycosidases. Fig. 4*a* reveals the



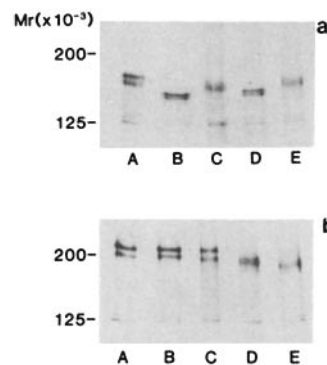


FIGURE 4. Endoglycosidase treatment of the 2H4 antigen. Con A-stimulated cells were labeled with [ $^{35}$ S]cysteine, methionine for a period of 10 min (a), or for a period of 10 min followed by a chase period of 100 min (b) before immunoprecipitation and digestion with various endoglycosidases. (A) Untreated 2H4 antigen; (B) endo H digestion; (C) neuraminidase digestion; (D) endo F digestion; (E) *O*-glycanase (and neuraminidase) digestion.

pattern of enzyme-treated 2H4 subunits derived from Con A-stimulated T cells that had been labeled with [ $^{35}$ S]cysteine, methionine for 10 min (chase period 0). Under these conditions, endo H caused a reduction in the  $M_r$  of the  $175/185 \times 10^3$  doublet to a doublet at  $150$  and  $160 \times 10^3$  (lane B), an  $M_r$  similar to that obtained from tunicamycin-treated cells (Fig. 3A, last lane). Endo H cleaves only the immature form of *N*-linked oligosaccharides (38). Likewise, endo F, which cleaves both the immature and mature forms of *N*-linked glycans (39), reduced the size of the  $175/185 \times 10^3$  to the lower  $M_r$  form (lane D). These data indicate that the 2H4 subunits at  $175$  and  $185 \times 10^3 M_r$  are derived from precursor forms at  $150$  and  $160 \times 10^3$  by the addition of multiple asparagine-linked oligosaccharides in an immature, polymannose form. In contrast, neither of the  $175$  or  $185 \times 10^3 M_r$  subunits possesses sialic acid or *O*-linked oligosaccharides as shown by the inability of neuraminidase (lane C) or the combination of neuraminidase and *O*-glycanase to significantly alter the molecular size of these subunits (lane E; the slight reduction in  $M_r$  is due to the curvature of the gel). Neuraminidase removes sialic acid from carbohydrate units, while *O*-glycanase will cleave *O*-linked oligosaccharides after the removal of sialic acid (40). Lastly, none of the glycosidases had an effect on the size of the 2H4 subunits at  $125,000 M_r$ , indicating the absence of *N*-linked or *O*-linked oligosaccharides.

Fig. 4b reveals the pattern of 2H4 subunits derived from Con A-stimulated cells after a 180-min period of chase and digested with various glycosidases. The anti-2H4 antibody precipitated two subunits at  $200$  and  $220 \times 10^3 M_r$ , in addition to the subunit at  $125 \times 10^3$ . Endo H had little if any effect on the positions of the  $200$  and  $220 \times 10^3 M_r$ , indicating the absence of immature *N*-glycans on these subunits (lane B). In contrast, endo F was found to reduce the  $M_r$  of these polypeptides by  $25$ – $30 \times 10^3 M_r$  (lane D); a change in  $M_r$  similar to that observed with the removal of *N*-linked oligosaccharides from the  $175$  and  $185 \times 10^3 M_r$  subunits (Fig. 4a, lanes B and D). Such data indicate that the majority of *N*-linked oligosaccharides or the 2H4 subunits at  $200 \times 10^3 M_r$  and  $220 \times 10^3 M_r$  are converted to a complex variety.

Digestion with a combination of neuraminidase and *O*-glycanase caused a further reduction in the size of the high  $M_r$  forms of the 2H4 antigen to a version at  $\sim 170$ – $180 \times 10^3 M_r$  (lane E). The use of neuraminidase without *O*-glycans also caused a reduction in  $M_r$  of the  $200$  and  $220 \times 10^3 M_r$  chains (lane C). The presence of sialic acid is consistent with the presence of complex *N*-linked glycans

TABLE I  
Blocking of AMLR/Con A-induced Suppressor/Inducer function by T200 Family of mAbs

Exp.	Activated T cells added	Antibody treatment	IgG production			
			Exp. 1	Suppression*	Exp. 2	Suppression*
			ng/ml	%	ng/ml	%
A <sup>‡</sup>	—	—	12,400	—	14,400	—
	A	5 × 10 <sup>5</sup>	8,600	31	9,200	36
		1 × 10 <sup>4</sup>	4,900	60	6,100	58
	B	5 × 10 <sup>5</sup>	12,900	0	15,200	0
		1 × 10 <sup>4</sup>	11,600	6	13,100	9
	C	5 × 10 <sup>5</sup>	8,400	32	8,700	40
		1 × 10 <sup>4</sup>	5,100	59	5,800	60
	D	5 × 10 <sup>5</sup>	8,100	35	9,100	37
		1 × 10 <sup>4</sup>	4,500	64	5,600	61
B <sup>‡</sup>	—	—	9,600 <sup>†</sup>	—	11,600	—
	A	5 × 10 <sup>5</sup>	5,900	39 <sup>†</sup>	7,700	34
		1 × 10 <sup>4</sup>	2,400	75	3,300	72
	B	5 × 10 <sup>5</sup>	8,900	7	10,700	8
		1 × 10 <sup>4</sup>	7,600	21	9,700	16
	C	5 × 10 <sup>5</sup>	6,200	35	7,600	34
		1 × 10 <sup>4</sup>	2,100	78	3,100	73
	D	5 × 10 <sup>5</sup>	5,700	41	7,400	36
		1 × 10 <sup>4</sup>	2,600	73	2,900	75

\* Percent suppression = 100 × [(Control IgG response) - (observed IgG response)] / (control IgG response).

<sup>‡</sup> T4 cells on day 7 activated in AMLR were treated with mAbs (1:100 dilution) at 4°C for 1 h without complement, washed extensively, and then added to 10<sup>5</sup> freshly isolated autologous PBL with PWM. These cells were cultured for 7 d, and IgG in culture supernatants was determined by RIA.

<sup>†</sup> T cells activated with Con A for 2 d were treated with mAbs (1:100) at 4°C for 1 h without complement, washed four times, and then added to 10<sup>5</sup> freshly isolated autologous PBL at the initiation of PWM-stimulated IgG synthesis cultures.

<sup>†</sup> Results were expressed as mean of triplicate samples. SEM was always <10%.

and O-glycosylation. However, the presence of O-glycanase caused a further reduction in size of some 30–40 × 10<sup>3</sup> M<sub>r</sub> (lane E). Thus, the presence of both N-linked and O-linked oligosaccharides account for some 60–70 × 10<sup>3</sup> M<sub>r</sub> on each of the 2H4 subunits. As previously seen, none of these enzymes had an effect on the 2H4 polypeptides at 125 × 10<sup>3</sup>. Collectively, these data indicate that the 2H4 subunits (with multiple immature N-linked glycans) at 175 and 185 × 10<sup>3</sup> M<sub>r</sub> are converted by the maturation of the N-linked oligosaccharides and the addition of O-linked oligosaccharides to the surface form at 200 and 220 × 10<sup>3</sup> M<sub>r</sub>.

*Anti-2H4 Antibody Exhibits Specificity in the Blockage of Suppression.* Given the reactivity of the anti-2H4 antibody with a subset of L-C/T200 antigens, it is of interest to determine whether the anti-2H4 and anti-L-C/T200 antibodies were capable of blocking the ability of T4<sup>+</sup>, 2H4<sup>+</sup> to induce suppression of Ig production by B cells. Previous studies (9) had shown that the pretreatment of AMLR-activated T4<sup>+</sup> cells with the anti-2H4 antibody abrogated the ability of these cells to induce suppression of Ig production by B cells. Table I shows the effect on suppression of pretreating AMLR-activated T4<sup>+</sup> cells or Con A-activated T lymphocytes with the anti-2H4 antibody and the other antibodies PD7/26, 2B-11, and 9.4 which are broadly reactive with the L-C/T200 antigens. The T cells were incubated with the various mAbs for 1 h at 4°C, washed extensively, and then added to a PWM-driven B cell assay. Significantly, the anti-2H4 antibody caused a marked effect in blocking the ability of the T cells to induce the suppression of Ig production by B cells. However, none of the antibodies that

were broadly reactive with the L-C/T200 antigens were found to have an effect. Thus, a correlation may exist in the specificity of recognizing an epitope on the L-C/T200 antigens at 200 and/or  $220 \times 10^3 M_r$  and the ability of antibody to interfere with the induction of immune suppression.

### Discussion

The 2H4 and 4B4 antigens define reciprocal subsets of T cells involved in providing suppressor inducer function ( $T4^+, 2H4^+$ ) and helper function ( $T4^+, 4B4^+$ ) to B cells (4, 5). In addition, the response of T lymphocytes to soluble antigen resides primarily within the  $T4^+, 2H4^-$  ( $T4^+, 4B4^+$ ) subset (9). In this study, we have defined the structure of 2H4 antigen in an effort to establish the molecular basis of 2H4 function. Importantly, the division of  $T4^+$  cells into these discrete subsets appears to be correlated to the differential expression of the 200 and  $220 \times 10^3 M_r$  forms of the L-C/T200 antigens on the surface of T cells (Fig. 1B). The  $T4^+, 2H4^+$  cells express at least four different forms of the T200 antigens at  $190$ – $220 \times 10^3 M_r$ , while in contrast, the  $T4^+, 2H4^-$  subset is restricted in expressing the isoforms at 180 and  $190 \times 10^3 M_r$ . Thus, by reacting with only the two high  $M_r$  versions of the L-C/T200 antigens, the anti-2H4 antibody has been able to distinguish two functionally discrete subsets of T cells. Immunodepletion experiments and peptide fingerprint analysis (data not shown) confirmed that the 2H4 chains at 200 and  $220 \times 10^3 M_r$  bands are analogous to members of the L-C/T200 family of antigens. This pattern was clearly different from that precipitated by the anti-4B4 antibody that reacted with a spectrum of polypeptides at 135, 160, and  $185 \times 10^3 M_r$  (Fig. 1a, lane E). None of these subunits were found to comigrate with the 2H4 bands or any members of the L-C/T200 antigens. Preliminary data indicate that the 4B4 structure is a member of the fibronectin receptor/VLA family of adhesion molecules (Rudd, C. E., et al., manuscript in preparation).

The presence of the multiple forms of the 2H4 antigen could have been the result of differences in glycosylation, as described for a version of the T200 antigen on cytotoxic T cells (18), or due to the synthesis of distinct polypeptides. Indeed, biosynthetic labeling over a prolonged period revealed a heterogeneous collection of bands precipitated by the anti-2H4 and 9.4 antibodies (Fig. 2). Pulse-chase analysis in conjunction with tunicamycin treatment clearly indicated that the 2H4 polypeptides correspond to the highest  $M_r$  isoforms of a pool of at least five distinct L-C/T200 antigens. This level of diversity is consistent with the structural variants of the polypeptide as derived from distinct mRNAs (21–23). However, at the protein level, this degree of diversity is greater than previously noted (18, 20). Although our cultures are enriched for T lymphocytes (>95% E rosette-positive), the presence of some 2–3% adherent cells could contribute to the heterogeneity of the different isoforms. Each of the 2H4 antigens at 200 and  $220 \times 10^3$  represent the highest  $M_r$  forms as derived from the pool of nascent L-C polypeptides (Fig. 3B). Thus, the anti-2H4 antibody reacts with the protein backbone of these isoforms of the T200 antigen. Tunicamycin treatment and digestion with endo H showed that these polypeptides are then converted by the addition of some 12–14 immature N-linked oligosaccharides to a doublet at 175 and  $185 \times 10^3 M_r$ . This number of glycans is in

agreement with the DNA sequence of the L-C/T200 antigen (13) and for other members of the LCA family (10, 18).

By some 20–30 min, the  $175/185 \times 10^3$  doublet is converted by the maturation of the *N*-linked glycans and the addition of numerous *O*-linked oligosaccharides to the surface form at  $200$  and  $220 \times 10^3$  (Figs. 3 and 4). Most, if not all, *N*-linked glycans are converted to an endo H-resistant complex form (Fig. 4B, lane B); a result that is in agreement with processing of other forms of the T200 antigens (19). Digestion with endo F revealed that this processing did not change significantly the contribution in molecular size of the *N*-glycans to the 2H4 isoforms (Fig. 4B, lane D). The major shift in the  $M_r$  of the  $175/185 \times 10^3 M_r$  polypeptides to the  $200/220 \times 10^3$  polypeptides is due principally to the addition of multiple *O*-linked oligosaccharides, as revealed by the ability of *O*-glycanase to reduce the molecular size by some  $30\text{--}40 \times 10^3 M_r$  (Fig. 4B). It is also apparent that at this stage in biosynthesis, the change in size of the 2H4 polypeptides was greater than that observed for the lower  $M_r$  isoforms of the L-C/T200 antigens (Fig. 3B). The 2H4 epitope is thus probably present on a stretch of amino acids that is unique to the  $200/220 \times 10^3 M_r$  isoforms of the L-C antigens and is enriched for *O*-linked oligosaccharides. Such a region appears to be localized toward the  $\text{NH}_2$ -terminal portion of the T200 antigen as deduced from cDNA clones encoding the human T200 antigens (21, 22). A similar region of the L-C antigen in rat may be recognized by an antibody termed OX22 (20).

However, despite certain similarities, important differences exist in the nature of the polypeptides recognized by the anti-2H4 and other anti-L-C/T200 antibodies that can identify subsets of T cells. One antibody, NK-9, which reacts specifically with human T cells appears to recognize three isoforms at  $190/200/220 \times 10^3 M_r$  (24). Another antibody, OX-22, that also reacts with the isoforms at  $190/200/220 \times 10^3 M_r$  appears to designate a subset of rat T lymphocytes that can provide help to T cells (18, 41). Thus, the ability of the anti-2H4 antibody to react with a more restricted number of T200 isoforms at  $200/220 \times 10^3$  may be correlated to its recognition of a different subset of T cells. The NK-9 antibody has been reported to react with 85–95% of T cells, while the OX-22 antibody reacts with some 75% of the rat T cell population (5, 19, 20). In contrast, the anti-2H4 antibody has been found to react with only 40–50% of T cells. Another antibody reactive with cytotoxic T cells appears to react with several subunits at  $190/200/220/240 \times 10^3 M_r$  (17, 18). In this case, the epitope recognized by this antibody is present on the carbohydrate units of the antigen, a situation different from the 2H4 epitope that is present on the protein backbone (Fig. 3). Such data argue for the unique nature of the 2H4 antigen, and support the view that distinct epitopes on the different isoforms of the T200 antigen may allow the identification of functionally discrete populations of lymphocytes.

The 2H4 antigen is also unique in its association with an additional nonglycosylated polypeptide at a  $125 \times 10^3 M_r$  subunit which was not apparent in immunoprecipitates formed by any of the anti-L-C/T200 (Fig. 2D vs. Fig. 2, B and C). This form of the 2H4 antigen was expressed as a single polypeptide, or as two or three subunits, depending on the experiment and donor (Fig. 2 vs. Fig. 4). However, in either case, the subunits shared the common feature of the

absence of *N*-linked or *O*-linked oligosaccharides as assessed by pulse chase and endoglycosidase digestion (Figs. 3 and 4). Further, this subunit was not detected at the cell surface as assessed by lactoperoxidase-catalyzed iodination (Fig. 1A, lane C). Whether this is related to a lack of accessible tyrosine/histidine residues, or to the absence of this subunit on the surface of cells remains to be established. It is unlikely that the  $125 \times 10^3 M_r$  subunit is a secreted protein since it underwent no significant change in intensity over the chase period of 20–100 min, as expected for a secretory polypeptide such as Ig (42). Furthermore, the subunit was not detected in the media of biosynthetically labeled cells (data not shown). The cellular location of this polypeptide, and its relationship to the bands at  $200/220 \times 10^3$  is presently being investigated.

The ability of the antibody to the 2H4 epitope on the  $200/220 \times 10^3 M_r$  chains to abrogate the function of the  $2H4^+$  T cells supports the notion that these T200 isoforms, and in particular the 2H4 epitope, may play a role in the regulation of suppressor/inducer function (Table I). The specificity of this inhibition was shown by the inability of the other three antibodies that were broadly reactive with L-C/T200 antigens to influence the suppressor/inducer function of the cells. Furthermore, the anti-2H4 antibody has not been found to block any other immune functions such as cytotoxicity and proliferation (data not shown). The T200 antigen may therefore act as receptor in regulating specific immune functions of subsets of cells after the initial stimulation of the T cell. The ability of another antibody against the p220 form of the T200 antigen to influence the proliferation of T cells to PHA is consistent with this notion (24). The nature of the intracellular signals generated by the 2H4 antigen, and its association with other receptors on the surface of T lymphocytes is presently being investigated.

### Summary

The T4 (CD4) subset of T lymphocytes has been subdivided into two major subsets, a suppressor/inducer subset ( $T4^+, 2H4^+$ ) and a helper subset ( $T4^+, 2H4^-$ ) on the basis of the differential expression of the L-C/T200 (CD45) antigens. The 2H4 antigen itself comprises at least three distinct polypeptides at 125, 200, and  $220 \times 10^3 M_r$ , of which the 200 and  $220 \times 10^3 M_r$  polypeptides constitute the highest  $M_r$  isoforms of a pool of five distinct L-C/T200 antigens. The  $T4^+, 2H4^+$  subset expresses at least four of these isoforms at 180, 190, 200, and  $220 \times 10^3$  on the cell surface, while the  $T4^+, 2H4^-$  subset expresses only the 180 and  $190 \times 10^3 M_r$  forms. Pulse-chase analysis and endoglycosidase treatment revealed that the  $125 \times 10^3 M_r$  chain of the 2H4 antigen is nonglycosylated, while the 200 and  $220 \times 10^3$  polypeptides are structurally related and derived by *N*- and *O*-linked glycosylation from two nascent subunits at 150 and  $160 \times 10^3 M_r$ . The function of the  $T4^+, 2H4^+$  subset could be blocked only by an antibody reactive with the L-C/T200 isoforms enriched with *O*-linked oligosaccharides at 200 and  $220 \times 10^3 M_r$ .

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