

DY DETERMINANTS, POSSIBLY ASSOCIATED WITH NOVEL  
CLASS II MOLECULES, STIMULATE AUTOREACTIVE CD4<sup>+</sup>  
T CELLS WITH SUPPRESSIVE ACTIVITY

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The human MHC comprises loci encoding at least three main segregant series of polymorphic HLA class II products, HLA-DR, -DQ, and -DP. These appear to have broadly similar functions, at least in vitro, since all can serve as restriction elements for T cell antigen recognition (1–3), as alloantigenic targets for T cell-mediated cytotoxicity (4–6), and as stimulatory antigens for lymphoproliferative responses (6). Quite probably, not all class II products have even now been identified and characterized at the protein level (7). Further, the presence of additional class II genes, *DOβ* and *DZα*, has also been reported (8, 9). No surface products of these genes have yet been identified, although they are expressed at the mRNA level (8, 9). The existence of more class II molecules derived by transcomplementation (10) or present as mixed isotypes (11, 12) is also to be expected.

mAbs reacting specifically with DR, DQ, or DP molecules have proven critical in investigating the structure and function of class II moieties. Moreover, mAbs reacting with epitopes broadly distributed on products of more than one locus may be informative in the definition of potential novel class II molecules (7). Using mAb TÛ39, which appears to possess such a uniquely broad reactivity, leukemias of different lineages were often shown to contain a much lower percentage of DR<sup>+</sup>, DP<sup>+</sup>, and DQ<sup>+</sup> cells than of TÛ39<sup>+</sup> cells (13, 14). This extra reactivity could be interpreted as reflecting the reactivity of mAb TÛ39 with class II molecules additional to the established DR, DQ, or DP series.

In a complementary approach, the identification of novel class II antigens may be accomplished by using cloned lines from in vitro primings between allogeneic donors matched for established class II specificities, and by blocking their stimulation with class II-specific mAbs. HLA-DP antigens were first demonstrated with such a primed lymphocyte typing (PLT)<sup>1</sup> technique (15). Using a

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<sup>1</sup> *Abbreviations used in this paper:* B-LCL, B-lymphoblastoid cell line; LAD, lymphocyte-activating determinant; LP, lymphocyte proliferation; PLT, primed lymphocyte test; SA, suppressive activity; SACI-RAM, *Staphylococcus aureus* Cowan I coupled to rabbit anti-mouse Ig; SC, suppressive cell; TCC, T cell clone.

similar rationale we previously reported (16) apparently novel lymphocyte-activating determinants (LADs) showing little polymorphism in the population, whose stimulation could be blocked by mAb TŪ39, but not by a number of other less broadly reactive class II-specific mAbs. These LADs have been operationally designated DY (14). The CD4<sup>+</sup> cloned lines responding to DY LADs exhibited weak and poorly biphasic proliferative responses not associated with the presence of any identifiable HLA specificity on the stimulating cells (16). Such DY-reactive T cell clones (TCC) were also unusual in their ability to inhibit proliferative responses of other lymphocytes in an apparently HLA-unrestricted fashion (16). In the present report it is shown that cells stimulated by DY determinants display autoreactivity and could therefore constitute a self-maintaining circuit with suppressive activity for lymphocyte proliferation (LP). Stimulation of LP and induction of suppressive activity via DY is shown not to be blocked by mAbs specific for DR, DP, or DQ, but is blocked by mAb SG520 and TŪ39. The latter mAb is shown to precipitate a putative novel class II-like non-DR, -DQ, -DP molecule, which is therefore a good candidate for the structure bearing DY determinants.

### Materials and Methods

*Lymphocyte Priming.* Peripheral blood mononuclear cells (PBMC) from donors typed for HLA-A, B, C, DR, Dw, DQw, and DPw antigens were isolated by density gradient centrifugation. Stimulator cells were  $\gamma$ -irradiated at 20 Gy and mixed with unirradiated responding cells in equal amounts at 10<sup>6</sup> cells/ml. Priming cultures were performed between DR/Dw and DQ phenotypically matched donors in 16-mm-diameter cluster plate wells (Costar, Cambridge, MA) or 25-cm<sup>2</sup> tissue culture flasks (Falcon Labware, Oxnard, CA) in medium consisting of RPMI 1640 + 25 mM Hepes supplemented with 10% heat-inactivated pooled nontransfused male serum, with antibiotics. After 6 d, cultures were supplemented with 20% vol/vol of conditioned medium (Lymphocult T; Biotest, Frankfurt, Federal Republic of Germany) to a final concentration equivalent to 20 U/ml of IL-2. After 10 d, the cultures were harvested, washed, and restimulated with twice their number of cells from the original stimulating cell donor, in fresh medium supplemented with Lymphocult T. After a further 4 d, PLT reagents were harvested and cryopreserved for use in PLT tests.

*Cloning and Cell Line Propagation.* PLT cells were restimulated with the original priming cells in Costar wells in medium supplemented with 20% of Lymphocult T, using 2  $\times$  10<sup>5</sup> primed cells plus 4  $\times$  10<sup>5</sup> stimulators in 2 ml medium with IL-2 at 20 U/ml. After 4 d, cells were cloned at limiting dilution and cultured by intermittent feeding with fresh medium (every 2–4 d) and periodic restimulation with specific stimulator PBMC (every 7 d). Limiting dilution was performed in 1-mm diameter culture wells, seeding 0.3–0.45 cells/well with 10<sup>4</sup> 30 Gy irradiated PBMC stimulator cells. Control plates containing 4.5 and 45 cells/well were set up to ensure single-hit characteristics of the limiting dilution curves ( $r > 0.9$ ). Contents of wells containing growing cells were transferred at 7–10 d to 7-mm diameter culture wells with 10<sup>5</sup> stimulator cells and fresh medium. After a further 3–5 d, cells were transferred to Costar wells, where they were maintained as above, using pooled lymphocytes from at least 20 donors as stimulators.

*PLT Restimulation.* Primed cells, cultured cells, and cloned cells were restimulated in U-well microtiter plates, generally using 10<sup>4</sup> responders and 10<sup>5</sup> stimulators (PBMC) per well. B-lymphoblastoid cell lines (B-LCL) and cloned TCC stimulators were used at lower cell numbers. PBMC and TCC were irradiated at 20 Gy and B-LCL at 80 Gy. Culture kinetic was varied. Medium was RPMI, 25 mM Hepes, 10% human serum, and antibiotics. 37 kBq/well [<sup>3</sup>H]TdR (sp act, 185 GBq/mmol; Amersham Corp., Arlington Heights, IL) was added 18 h before termination of the cultures.

**Quantification of IL-2 Secretion.** Supernatants of specifically stimulated T cell clones were prepared by incubating  $4 \times 10^5$  cloned cells with  $5 \times 10^5$  80 Gy-irradiated B-LCL in 2 ml of medium containing 1% HS for 48 h. To quantitate IL-2 production, supernatants were titrated onto IL-2-dependent T cell lines and [ $^3\text{H}$ ]TdR incorporation was measured after 24 h. Highly purified natural IL-2 (Lymphocult T-HP; Biotest) was used as a positive control, and Probit analysis was applied on an IBM-PC (program by Blaurock, M., G. Pawelec, and P. Wernet, submitted for publication) to calculate units of IL-2 with reference to the International Union of Immunological Societies-Biological Response Modifiers Program (IUIS-BRMP) standard.

**mAb Inhibition of Stimulation.** mAbs were added to restimulation assays at the initiation of culture and remained present for the duration of the assay. Stimulating cells were plated first, followed by mAbs, and lastly, after a short pause, the responding cells were plated. Previous experiments with the Tübingen series mAbs used here (TÜ22, anti-DQ; TÜ34, TÜ37, anti-DR; and TÜ39, binding the products of at least DR and DP) had provided no evidence of stimulation-inhibition mechanisms divorced from effects of the mAbs binding to the stimulating cells (17, 18). Nonetheless, in certain experiments, stimulating cells were pretreated with mAbs by incubating in undiluted hybridoma supernatant at 4°C for 1 h, followed by washing three times in cold medium, and immediate addition to culture wells already containing the responders. B-LCL used as stimulators had been shown by FACS analysis to bind strongly the mAbs used in the blocking experiments, although the level of expression of DQ and DP antigens was almost always lower than that of DR antigens (data not shown). TÜ mAbs (13, 14, 17-20) were used as tissue culture supernatants (25%, 1-3 µg/ml), other mAbs as a 1:100 dilution of ascites. Additional mAbs used were as follows: L243 (6), Q2/70 (18, 20), and SG157 (18) specific for HLA-DR (although Q2/70 may also bind DQ, reference 20); SPV-L3 (4) and Leu10 (18) specific for DQw and DQw1,3 molecules, respectively; B7/21, specific for HLA-DP (5, 15); and PL5 (6), DA6.231 (6, 18), and SG520 (6, 18) "broadly" reactive with multiple class II molecules. mAb B7/21 (anti-FA) ascites was a kind gift of F. Bach, Immunobiology Research Center, Minneapolis, MN, and the B7/21 hybridoma was a generous gift of I. Trowbridge, Salk Institute, San Diego, CA; SPV-L3 was from J. de Vries and H. Spits, UNICET, Dardilly, France; SG157 and SG520 came from S. Goyert, Hospital for Joint Diseases, New York; DA6.231 was from K. Guy, MRC Clinical and Population Cytogenetics Unit, Edinburgh, Scotland; Q2/70 was from S. Ferrone, New York Medical College, New York; PL5 was from R. Knowles, Memorial Sloan-Kettering Cancer Center, New York; and Leu10 and L243 were from Becton Dickinson & Co., Mountain View, CA.

**Sequential Immunodepletion Procedures.** For immunoprecipitation studies, mAbs L243, TÜ22, B7/21, and TÜ39 were purified by protein A-Sepharose 4B affinity chromatography according to Ey et al. (21).

TCC were biosynthetically labeled by resuspending  $4 \times 10^7$  exponential growth phase cells in 4 ml MEM without L-methionine. The medium was supplemented with 5% methionine-free (dialyzed three times) human serum and 20 U/ml IL-2, and, after 1 h at 37°C, with 18,500 kBq of [ $^{35}\text{S}$ ]methionine ( $3.7 \times 10^{10}$  Bq/mmol; Amersham Corp.). After a further 4-h incubation, radiolabeling was terminated by washing the cells three times with cold saline 0.02% sodium azide, and 1 mg/ml L-methionine. Washed cells were resuspended in 2 ml of NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.02% azide, and 1 mM PMSF), and incubated for 30 min on ice. Insoluble material was removed by centrifuging at 10,000 g for 30 min. Cell extracts were loaded onto a Lens culinaris affinity column for glycoprotein purification according to Hayman and Crumpton (22). In a second series of experiments,  $2 \times 10^7$  washed TCC were surface labeled with  $^{125}\text{I}$  by the lactoperoxidase catalyzed method (23). Briefly, TCC were resuspended in 0.5 ml of cold saline to which 18,000 kBq of carrier-free sodium  $^{125}\text{I}$  (Amersham Corp.) was added. In rapid sequence, 30 U of lactoperoxidase (sp act, 90 U/ml; Calbiochem-Behring Corp., San Diego, CA), 15 µl of glucose oxidase (Sigma Chemical Co.), and 75 µl of glucose (50 mg/ml, Sigma Chemical Co.) were added, and the reaction was stopped after 30 min by repeated washing in cold saline.

Immunoprecipitation studies were performed as previously described (24). Briefly, labeled cell extracts were precleared with 100  $\mu$ l of protein A-bearing *Staphylococcus aureus* Cowan I (SACI) and 50  $\mu$ l of SACI coupled to rabbit anti-mouse globulin (SACI-RAM).  $2 \times 10^6$  cell equivalents were then incubated with 1  $\mu$ g of protein A-Sepharose-purified mAbs at 4°C for 1 h. Immunocomplexes were then incubated with 50  $\mu$ l SACI-RAM to ensure identical binding capacity of the SACI to the various mAbs. The precipitates were then washed three times in lysis buffer without detergent. For sequential immunoprecipitations, the supernatant was retained for serial transfer at each step to tubes containing identical amounts (1  $\mu$ g) of the different mAbs, followed by SACI-RAM, and repetition of the above. At each step, material was retained for gel analysis as follows: bound proteins were solubilized by boiling for 3 min in elution buffer containing SDS and DTT, and visualized by electrophoresis on 12.5% polyacrylamide slab gel (25) followed by fixing and fluorography in Amplify (Amersham Corp.) and autoradiography on Hyperfilm MP (Amersham Corp.) at -70°.

*Cell-mediated Cytotoxicity Assay.* Standard  $^{51}\text{Cr}$ -release assays were performed as previously described (26). Briefly,  $2\text{--}5 \times 10^6$  K562 line cells were incubated with  $3.7 \times 10^6$  Bq of sodium chromate (sp act, 22.2 GBq/mg  $^{51}\text{Cr}$ ; Amersham-Buchler, Frankfurt, FRG) in 0.4 ml culture medium for 90 min, washed three times, and cocultured for 4 h with effector cells. Supernatants were removed by pipette thereafter for gamma spectroscopy.

*Induction of Suppressive Activity by T Cell Clones.*  $2 \times 10^6$  PBMC were incubated for 3 d with  $10^5$  20 Gy-irradiated TCC in medium with 10% serum, without IL-2. Harvested cells were then cultured with 20 U/ml of IL-2 for at least 7 d before testing by titrating into MLC. To investigate blockade of SA induction with mAbs, 3-d PBMC + TCC cultures were performed in the presence of 25% supernatant or 1:100 diluted ascites. Cells were washed, cultured in IL 2 and then added to MLC. TCC cultured alone under these conditions did not survive, and PBMC cultured alone did not cause suppression.

## Results

*Restimulation of Clones Specific for DY Antigens Inhibited by mAb.* In three sensitization/cloning experiments with the same donors, ~60% of all derived clones showed autonomous proliferative capacity on alloantigen rechallenge. Of these, no more than 10% recognized LADs associated with the disparate DPw specificity of the stimulator, whereas proliferative responses of the remainder did not correlate with any DPw specificity. The majority of stimulators elicited either clearly positive or equivocal responses, as assessed by an objective cluster program. Even for the unequivocally positive responses, restimulation was not associated with the presence of any particular established class I or class II specificity (16). Surprisingly, the spontaneous [ $^3\text{H}$ ]TdR incorporation of the clones incubated with medium alone was considerably higher than that for representative PLT clones specific for DR-, DQ-, or DP-associated LADs. Moreover, PBMC of the autologous donor from whom the clones were derived further stimulated these clones, although all responses were very weak compared with DR-, DQ-, or DP-specific responses. This is illustrated for four DY-reactive clones designated 102-1, 102-3, 105-5, and 106-13 in Fig. 1. Titrating the stimulating cells (Fig. 1), or altering the kinetics of restimulation (data not shown), indicated that not only did these response patterns remain stable, but that autologous cells commonly stimulated more strongly than allogeneic cells. Similar results were obtained when B-LCL instead of PBMC were used as stimulators (Table I).

We attempted to characterize the stimulatory DY-LADs by blocking stimulation with mAbs. A number of DR and DQ mAbs, and one DP mAb, failed to

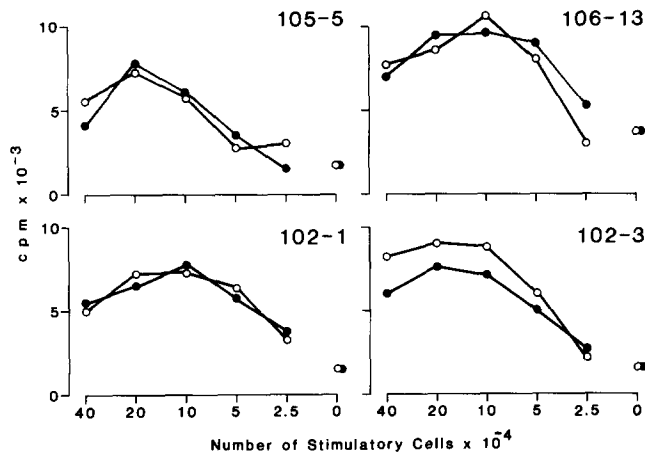


FIGURE 1. Autoreactivity of DY-specific TCC. Constant  $10^4$ /well TCC were stimulated by titrated amounts of irradiated PBMC from the same donor as the clones (open circles, autologous) or from the donor of the stimulating cells for the MLC from which the clones were derived (filled circles, specific). Results are shown as median cpm of triplicate cultures (SEM was <12%).

TABLE I  
DY Expression on B-LCL

Clone	Stimulator					
	Autologous		Allogeneic		Allogeneic	
	PBMC	B-LCL	PBMC	B-LCL	PBMC	B-LCL
102-1	6,353 ± 582*	8,972 ± 549	4,316 ± 335	5,291 ± 938	4,378 ± 327	5,911 ± 590
103-1	9,568 ± 720	7,623 ± 836	7,399 ± 877	8,443 ± 562	10,102 ± 991	9,628 ± 892
106-8	3,281 ± 156	3,697 ± 110	2,926 ± 126	3,468 ± 479	4,381 ± 200	6,528 ± 537
106-13	7,281 ± 881	9,428 ± 1,003	5,001 ± 323	5,575 ± 328	5,699 ± 686	6,023 ± 720

\* Data presented as mean cpm ± SEM of triplicates of  $10^4$  cloned cells per well stimulated with  $10^5$  PBMC or  $2.5 \times 10^4$  B-LCL cells.

block stimulation of DY-specific TCC by B-LCL, although each of the mAbs was capable of blocking stimulation of other clones via the appropriate class II type. Thus, the left hand panel of Fig. 2 shows the relative responses of the DY-specific clones 102-1, 103-1, and 106-8 stimulated in the presence of a range of class II-specific mAbs. For comparison, the right hand panel of Fig. 2 shows stimulation inhibition patterns of the same mAb for anti-DP clone 64-2, anti-DR clone 249-13, and anti-DQ clone 233-7. Essentially similar results were obtained by using PBMC or stimulatory TCC instead of B-LCL as stimulators (data not shown). Blocking of stimulation by a mixture of DR, DQ, and DP mAbs was also not seen (data not shown). However, the broadly reactive class II-specific mAbs TÛ39 and SG520, but not PL5 or DA6.231, did block stimulation by DY (Fig. 2). Additionally, TÛ39 reduced the level of [ $^3$ H]TdR incorporation to below that of clones incubated in medium alone. Inhibitory effects were not caused by nonspecific activity of the mAbs because in the presence of 20 U/ml of IL-2 proliferation was not affected by TÛ39 (Table II). Furthermore, pretreatment of the stimulating cells with mAb TÛ39 resulted in the retention of a degree of inhibition of stimulation (Table II).

*mAb TÛ39 Precipitates Class II-like Molecules Different from DR, DQ, or DP.* TCC were surface labeled with  $^{125}$ I and  $2 \times 10^6$  cell equivalents of soluble preparation were subjected to sequential immunoprecipitations designed to deplete molecules reacting with well-characterized mAbs specific for mono-

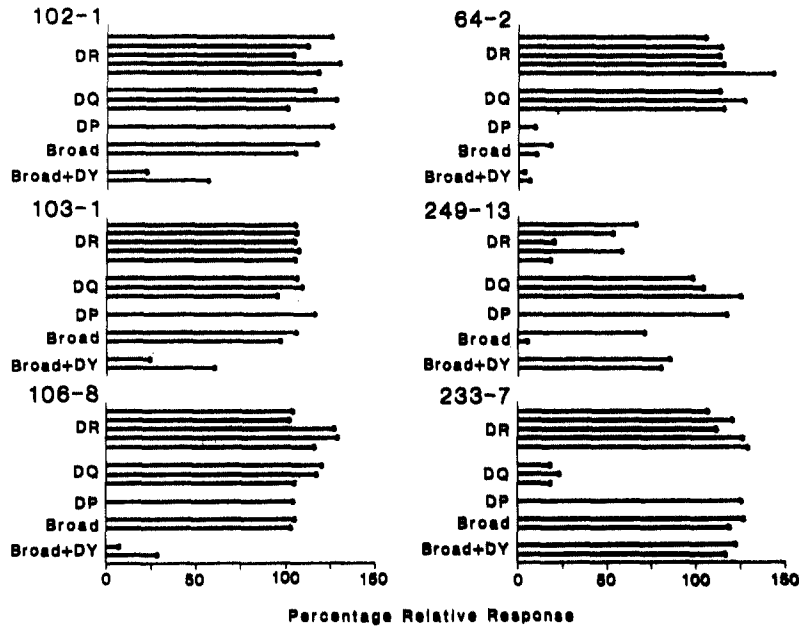


FIGURE 2. Inhibition of stimulation by mAbs. (Left) DY-specific TCC; (right) representative DP (64-2), DR (249-13), and DQ (233-7)-specific TCC. mAbs were as follows (from top to bottom): DR: TÛ34, TÛ37, L243, Q2/70, and SG157; DQ: TÛ22, SPV-L3, Leu 10; DP: B7/21; broad: PL5, DA6.231; broad + DY: TÛ39, SG520. Results are shown as percent relative response compared with the value in the presence of nonbinding control mAb (W6/32.HK).

TABLE II  
DY Stimulation Inhibited by mAb TÛ39

Stimulator	-	+	+	+	+	+
mAb TÛ39	-	-	-	+	+	+
IL-2	-	-	+	-	-	+
Clone <sup>§</sup>						
103-1	1,869 ± 230	6,292 ± 630	14,297 ± 1,501	2,496 ± 328	758 ± 114	13,634 ± 1,271
105-5	1,394 ± 101	6,180 ± 729	16,285 ± 1,499	1,981 ± 266	993 ± 82	15,200 ± 1,600
106-8	1,995 ± 204	9,456 ± 888	22,187 ± 3,003	3,053 ± 422	1,055 ± 236	21,864 ± 2,149

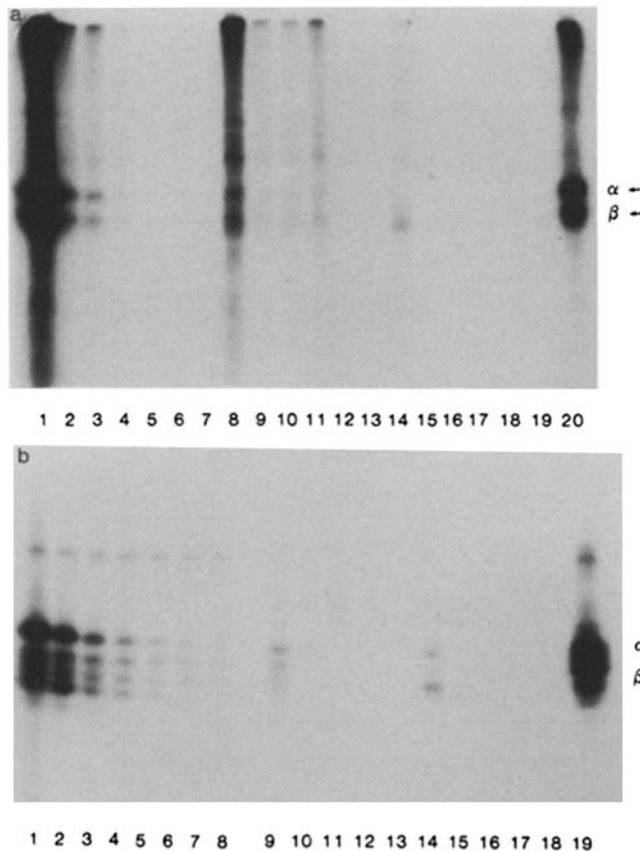
\* Stimulating cells pretreated with mAb TÛ39.

† MAb TÛ39 present for duration of coculture.

‡ 10<sup>4</sup> TCC were stimulated with 2.5 × 10<sup>4</sup> B-LCL cells (Stimulator +) or in medium alone (Stimulator -). Where IL-2 was present (IL-2 +), 20 U/ml was used. Where mAb TÛ39 was present (mAb TÛ39 +), 25% hybridoma culture supernatant was used.

morphic epitopes of DR, DQ, or DP molecules (Fig. 3a, lanes 1-20). Five sequential precipitations with mAb L243 (lanes 1-5) sufficed to remove all DR molecules (as shown by precipitation with SACI-RAM alone, lanes 6 and 7). This was followed on the same lysate by four precipitations with TÛ22 to remove DQ molecules (lanes 8-11, and SACI-RAM alone, lanes 12 and 13), and finally four sequential precipitations with B7/21 to remove DP molecules (lanes 14-17, SACI-RAM alone, lanes 18 and 19). Finally, lane 20 shows that mAb TÛ39 still precipitated heterodimeric molecules similar to class II products, even after DR, DQ, and DP molecules were depleted from the lysate.

To show that the "extra" TÛ39<sup>+</sup> molecules were indeed synthesized by the



**FIGURE 3.** (a) mAb TÛ39 detects molecules other than HLA-DR, -DQ, and -DP.  $2 \times 10^6$  cell equivalents of  $^{125}\text{I}$ -lactoperoxidase surface-labeled TCC lysates were subjected to sequential immunoprecipitation with class II-specific mAbs followed by SDS-PAGE: L243, lanes 1-5, and SACI-RAM alone, lanes 6 and 7; TÛ22, lanes 8-11, and SACI-RAM alone, lanes 12 and 13; B7/21, lanes 14-17, and SACI-RAM alone, lanes 18 and 19; TÛ39, lane 20. Relative positions of the  $\alpha$  and  $\beta$  chains are indicated, as compared with SDS-PAGE molecular mass standards. (b) TCC themselves synthesize TÛ39<sup>+</sup> "extra" class II-like molecules. Lysates of [ $^{35}\text{S}$ ]methionine metabolically labeled TCC were subjected to sequential immunoprecipitations and SDS-PAGE analysis: L243, lanes 1-6, and SACI-RAM alone, lanes 7 and 8; TÛ22, lanes 9-11, and SACI-RAM alone, lanes 12 and 13; B7/21, lanes 14-16, and SACI-RAM alone, lanes 17 and 18; TÛ39, lane 19.

TCC themselves, metabolic labeling with [ $^{35}\text{S}$ ]methionine, followed by similar sequential immunoprecipitation procedures, was undertaken. Results of one such experiment are shown in Fig. 3b. Six precipitations with L243 (lanes 1-6) removed DR molecules (as shown by precipitation with SACI-RAM alone, lanes 7 and 8); next, repeated precipitations with TÛ22 removed DQ molecules (lanes 9-11, SACI-RAM alone, lanes 12 and 13); after this, precipitations with B7/21 (lanes 14-16) removed all DP molecules (SACI-RAM alone, lanes 17 and 18). Finally, lane 19 shows that TÛ39 still precipitated a large amount of class II-characteristic two-chain heterodimers also from metabolically labeled TCC. Essentially identical results were obtained also with B-LCL (data not shown).

*Suppressive but not Helper T Cell Clones Stimulate DY-specific PLT Clones.* Since certain TCC as well as B-LCL expressed novel TÛ39<sup>+</sup> non-DR, -DQ, -DP molecules, they were tested for their expression of DY by using them as stimulators for PLT clones. It was found that only TCC that were suppressive for LP responses in MLC were able to stimulate DY-specific clones. Helper TCC (defined by their ability to help B cells to secrete Ig, data not shown) that failed to suppress LP also failed to stimulate these reagents (Fig. 4). Moreover, similar levels of restimulation responses were observed whether allogeneic or autologous stimulating cells were used. Thus, Fig. 4 shows the responses of the four DY-specific

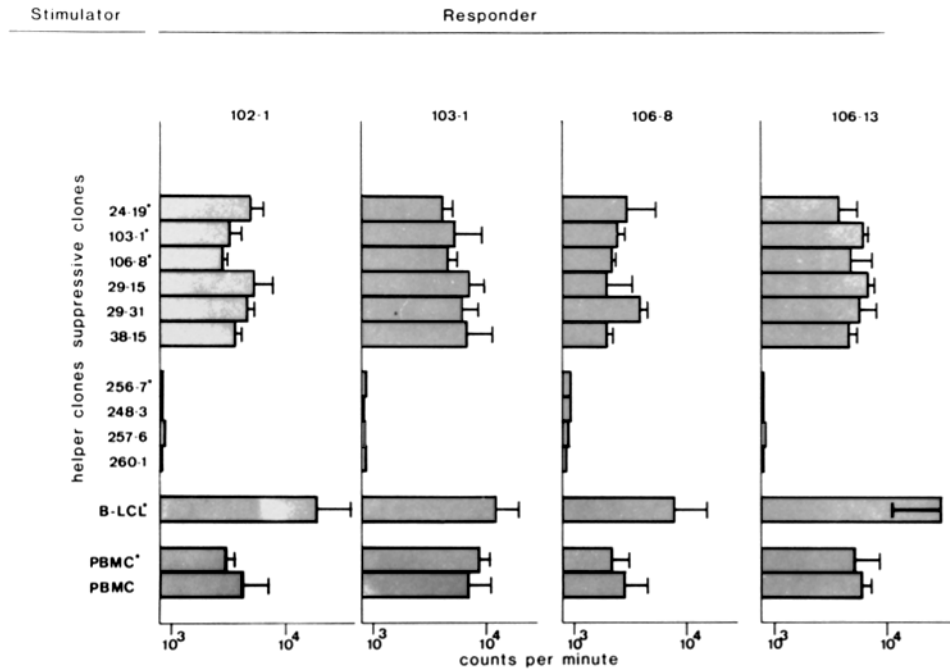


FIGURE 4. Cloned suppressive but not helper T cells stimulate DY-specific PLT clones. Data are presented as mean cpm of triplicate cultures  $\pm$  SEM, after subtraction of background (cpm of clone cultured in medium alone) of  $10^4$  responders stimulated with  $10^5$  PBMC or  $2.5 \times 10^4$  TCC or B-LCL for 66 h. The asterisk denotes that the stimulator cells were derived from the same donor as the responding cells.

PLT clones 102-1, 103-1, 106-8, and 106-13 rechallenged with a range of TCC, as well as with autologous and allogeneic PBMC and autologous B-LCL cells. From these results, the expression of DY on activated TCC would seem to correspond to their functional status. Remarkably, this also applied to the DY-specific clones themselves, which were found to be autostimulatory, and could respond to one another (Fig. 4). Such clones could be stimulated equally well by (irradiated) autochthonous cells as by cells from different clones.

*Functional Activity of DY Antigens.* Since only those TCC with suppressive activity were capable of stimulating DY-specific PLT clones, and since TCC responsive to DY were, unlike the majority of  $CD4^+$  PLT clones, themselves suppressive (16), it seemed likely that DY could be a major regulator of suppression. This possibility was further investigated in the following experiments. DY-specific clones 102-1, 103-1, 106-8, and 106-13 (Fig. 5, *top*) suppressed proliferation in allogeneic MLC practically as strongly as control suppressive clones 29-31 and 38-15 (Fig. 5, *bottom*). Helper TCC, on the other hand (DR5- and DPw3-specific clones 248-3 and 64-2 in Fig. 5), generally failed to suppress under these conditions. Moreover, other types of suppressive TCC, as well as the DY-reactive PLT clones themselves, were able to induce SA in normal PBMC, which was blocked by TÛ39 but not by anti-DR, -DQ or -DP mAb (26), indicating the involvement of DY in the generation of suppression. When PBMC were stimulated with DY<sup>+</sup> suppressive TCC (DY-specific PLT 106-8 or control



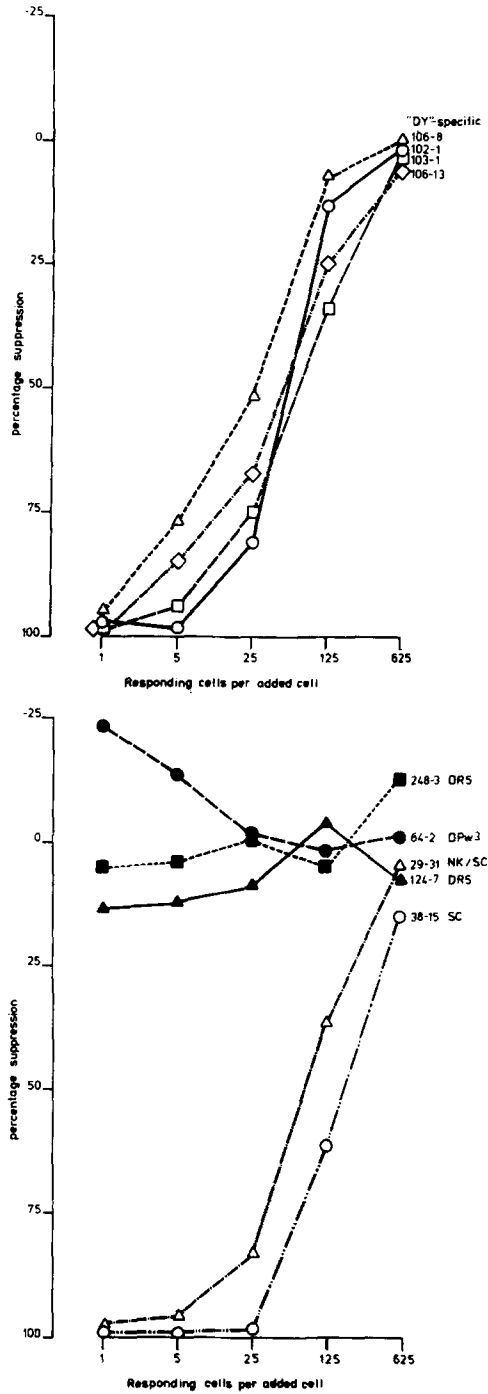


FIGURE 5. Suppressive activity in MLC of PLT clones specific for DY LADs. Cloned cells as shown were irradiated and titrated at the ratios shown directly into allo-MLC in which the responding cells were derived from a donor mismatched for MHC class I and II specificities with the donor of the clones. Results are expressed as percent suppression of the MLC performed in the absence of added suppressive cells.

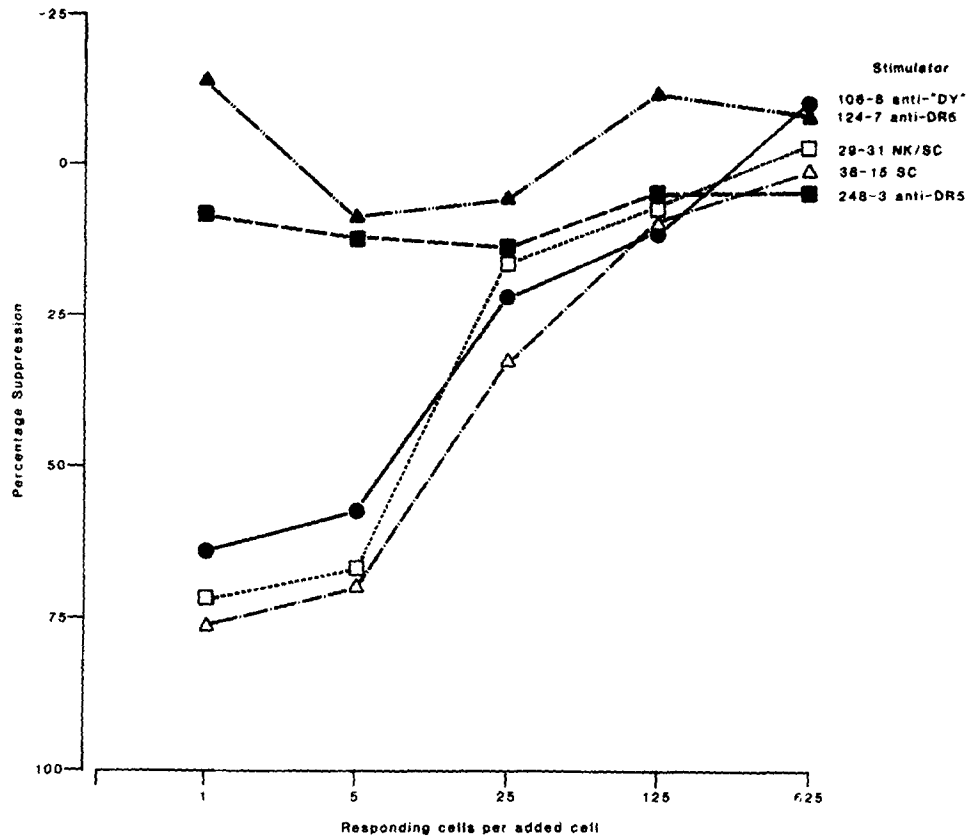


FIGURE 6. Suppressive activity in MLC of PBMC stimulated with suppressive clones. Allogeneic PBMC were cocultured for 3 d with the TCC shown (stimulator), followed by a further 7-d culture in purified IL-2, before irradiation and titration at the ratios shown into allo-MLC. Results are expressed as percent suppression of the MLC performed in the absence of added suppressive cells.

suppressible clones 29-31 and 38-15) for 3 d, followed by short-term culture in IL-2-supplemented medium, they were found to exert potent suppressive activity on LP responses in MLC (Fig. 6). In contrast, cell lines derived from PBMC stimulated with DR5-specific nonsuppressive helper TCC 124-7 or 248-3 failed to suppress (Fig. 6). In addition, to establish whether they recognized DY determinants, these lines were stimulated with PBMC and B-LCL in the absence of IL-2. Thus, PBMC were stimulated by irradiated suppressive clone 38-15 for 3 d, followed by propagation with IL-2 for 10 d, and were then restimulated with a range of different cells. They were found to proliferate in the presence of most stimulator cells, including PBMC from the autologous donor, GP (Fig. 7), as well as allogeneic PBMC and B-LCL (KR), and suppressive (29-31, 38-15) but not helper (248-3) TCC. This is a pattern characteristic of "DY"-reactive cells. Moreover, this stimulation was preferentially blocked by mAb TÛ39, but not TÛ22, TÛ34, or B7/21 (Fig. 7). In contrast, PBMC cultured under the same conditions with non-suppressive HLA-D-specific helper TCC responded weakly to specific allogeneic but not to autologous PBMC and B-LCL stimulators,

Responder: GP-PBMC and clone 38-15x for 3 days followed by 10 days culture with IL-2

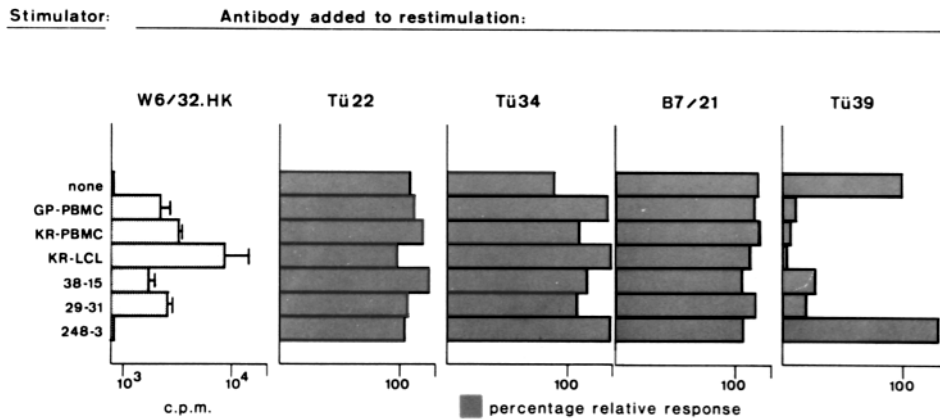


FIGURE 7. Lymphocytes primed against suppressive clones recognize DY LADs. PBMC were cocultured with irradiated DY<sup>+</sup> clone 38-15 cells for 3 d, followed by a further 10-d culture in IL-2. These cells were then restimulated for 66 h by the range of stimulating cells shown, in the presence of control mAb W6/32.HK (data presented in the left hand panel as mean cpm  $\pm$  SEM, after subtraction of the background) or, in the rest of the Figure, in the presence of the class II-specific mAb shown (data presented as percentage relative response compared with the value with W6/32.HK).

were unable to respond to autologous T cell clones, and were inhibited by anti-DR mAb (data not shown).

*No Further Functions of DY-specific Clones Found.* DY-specific PLT clones exhibited a peculiarly weak autonomous proliferative capacity, although they grew well when provided with exogenous IL-2. Increasing the amount of stimulating antigen available by using larger numbers of stimulating cells did not result in enhanced proliferative responses (Fig. 1). Consistent with their weak proliferative capacity, and unlike DR-, DQ-, or DP-specific PLT helper cells, DY-reactive cells secreted very modest amounts of IL-2 into the culture medium after stimulation (Table III). However, they were found to produce IL-2 with the same kinetic as other class II-reactive PLT clones, i.e., peaking at 36–48 h (data not shown). Despite their CD4<sup>+</sup>, Leu-8<sup>-</sup> phenotype (16), these suppressive TCC did not function as helpers for B cells (data not shown). Cytolytic activity of DY-specific TCC on NK-susceptible targets or on B-LCL was not measurable in the <sup>51</sup>Cr-release assay (16) even in the presence of PHA and/or 20 U/ml of IL-2 (Table IV).

## Discussion

After priming between HLA-DR/Dw and DQ-matched homozygous typing cells, a large proportion of derived clones was found to manifest weak autonomous proliferative activity against LADs widely distributed in the population (16). This peculiarly broad reactivity was not due to lack of monoclonality of the test reagents, because Southern blotting of their DNA with TCR- $\beta$  and - $\gamma$  chain probes indicated monoclonal rearrangements of these genes (27). Such clones appeared to be similar to most DR-, DQ-, or DP-specific PLT clones in that they

TABLE III  
*Secretion of IL-2 by PLT Clones*

Clone*	Specificity	Secreted IL-2 <sup>†</sup>
		U/ml
102-1	DY	2.1
102-3	DY	1.8
103-1	DY	4.3
105-5	DY	3.6
106-8	DY	5.0
64-2	DPw3	14.3
248-3	DR5	21.6
250-7	DR5	15.2
257-6	DR5	14.9
233-7	DQw3	16.4

\* Clones were specifically stimulated for 48 h before collection of supernatant for IL-2 assay.

<sup>†</sup> Quantification of IL-2 by Probit analysis compared with the IUIS-BRMP IL-2 standard, concentration expressed in units per milliliter.

TABLE IV  
*Lack of Cytotoxicity of DY-specific TCC*

Effector	E/T	Target K562 in the presence of:			
		Medium	1% PHA	20 U/ml IL-2	PHA + IL-2
103-1	50:1	0.5	-1.6	1.5	0.2
	17:1	-2.7	2.1	0.3	-1.9
105-5	100:1	3.1	-2.7	-2.3	0.6
	33:1	1.1	3.4	1.8	3.9
106-8	50:1	-6.7	-4.2	0.8	1.5
	17:1	-2.2	-1.3	1.7	2.2
PBMC	100:1	66.5	95.6	67.3	88.9
	33:1	48.0	65.1	43.7	52.4

$4 \times 10^3$  <sup>51</sup>Cr-labeled K562 cells were incubated for 4 h in the presence of TCC or PBMC at the E/T ratios shown. Medium was supplemented with 1% PHA and/or 20 U/ml of IL-2. Results are expressed as percent specific <sup>51</sup>Cr release (mean of triplicates, SEM <18%).

were CD4<sup>+</sup> and secreted IL-2, but they differed in that they exerted strong suppressive activity on LP responses (14, 16). Although it is thought unlikely that the suppressive activity could be due to cytotoxic effects, because these TCC showed no killing of sensitive target cells even in the presence of PHA and IL-2 (Table IV), it cannot be completely excluded that selective cytotoxicity on a minor population such as dendritic cells might contribute to inhibition of LP responses. However, the ability of these clones to induce suppressive activity in normal PBMC could not be explained in the same way. Others have also described CD4<sup>+</sup> T cells with suppressor/inducer capability, in freshly isolated populations. CD4<sup>+</sup>, 2H4<sup>+</sup> (CD45R<sup>+</sup>) T cells were previously identified as suppressors/inducers (28); however, CD45R is rapidly lost from TCC during culture, regardless of their function (29). Thus, the relationship between such CD45R<sup>+</sup> cells, and those described here, is not clear. DY-reactive SA-inducers are also negative for

another marker, Leu-8 (16), which is said to identify CD4<sup>+</sup> suppressor/inducer cells (30).

DY-specific TCC displayed an unusual type of autoreactivity, being restimulated not only by autologous PBMC or B-LCL, but also by HLA-mismatched allogeneic cells. Using comparable priming conditions, similar reactivities in uncloned PLT cells have been noted previously (31), and, more recently, Martin et al. (32) have described possibly similar findings at the clonal level (32). The LADs involved in stimulating autoreactive lymphocyte populations have been repeatedly shown to be, or to be associated with, class II molecules. Although FACS analysis showed that helper TCC expressed quantitatively similar amounts of DR, DQ, and DP antigens compared with the suppressive TCC (33), they differed in their ability to stimulate "DY"-specific clones (Fig. 4). This association of stimulatory activity with function of the clones surprisingly applied also to the autoreactive clones themselves, since these were mutually cross-stimulatory as well as autostimulatory. This implies that such cells are able to present DY LADs to each other. It is somewhat puzzling, therefore, that stimulation by autologous irradiated PBMC or autochthonous clones led to greater proliferation than observed in medium alone, since DY-reactive cells by themselves should constitute an autostimulatory circuit. Indeed, [<sup>3</sup>H]TdR incorporation by the clones in medium alone was higher than seen for DR-, DQ- or DP-specific clones, but was still higher in the presence of irradiated cells, even when these came from the same clone. In the case of autostimulatory PBMC or B-LCL, a more efficient antigen presentation, for example, by dendritic cells in the PBMC or by the LCL, than by the TCC themselves might help to explain this apparent anomaly. For self-stimulation by autochthonous clones, it is conceivable that the irradiated stimulator TCC themselves would also respond to unirradiated cells in the culture wells by secreting IL-2. This would amplify the response of the unirradiated cells, since one of the characteristics of the DY response is the very limited amounts of autocrine IL-2 production by stimulated cells (Table III).

The broad class II-reactive mAb TÛ39, unlike exclusively or preferentially DR-, DQ-, or DP-specific mAbs, blocked both LP stimulatory capacity and SA-inducing activity mediated by CD4<sup>+</sup> suppressive, but not by CD4<sup>+</sup> helper, TCC (26). The same pattern of blocking applied to stimulation of the autoreactive clones described here (Fig. 2). Various mAbs against HLA-DR, -DQ, and -DP failed to block stimulation of DY-specific autoreactive clones, whereas they could block stimulation of PLT clones by appropriate alloantigens. This is consistent with, but does not prove, the presence of DY determinants on molecules other than DR, DQ, or DP. That stimulation of autoreactive clones was blocked by two of four broadly reactive mAbs, SG520 and TÛ39 (Fig. 2), however, implies that these clones do recognize structures bound by certain class II-specific mAbs. Since mixtures of anti-DR, -DQ, and -DP mAbs also failed to block stimulation (34, and data not shown), it is improbable that the clones were capable of reacting to shared determinants on all these molecules, and therefore were not blocked by mAbs against any single one. Blockade of DY-specific TCC stimulation by TÛ39 was abrogated by exogenous IL-2 (Table II), suggesting that the mAb was not mediating a nonspecific inhibitory effect. Despite this, [<sup>3</sup>H]TdR incorporation was reduced by TÛ39 to below the level measured in medium alone.

The most likely explanation for this finding is that an autostimulatory circuit within the clone was broken by the binding of the mAb to DY antigens, resulting in blockade of DY-directed self-stimulation. The existence of an autostimulatory circuit within the clones would also help to explain why stimulation of DY-specific cells after pretreating only the stimulators with mAb TÛ39 was not reduced to background (Table II).

There is evidence that TÛ39 binds determinants other than those carried by DR, DQ, or DP molecules. First, studies of class II expression on leukemias and peripheral blood cells early after bone marrow transplantation demonstrated that certain DR<sup>-</sup>, DQ<sup>-</sup>, and DP<sup>-</sup> cells still reacted with mAb TÛ39, or that the proportion of TÛ39-reactive cells was much higher than the sum of DR<sup>+</sup>, DQ<sup>+</sup>, and DP<sup>+</sup> cells (13, 14). Second, preliminary biochemical analyses provided evidence consistent with TÛ39 reactivity on non-DR, -DQ, or -DP class II-like molecules of very similar molecular mass to DR molecules (Fig. 3). Thus, sequential immunoprecipitations to remove DR (and DR<sub>w</sub>, reference 35), DQ, and DP molecules from lysates of surface-labeled TCC failed to deplete all of the class II-like molecules with which mAb TÛ39 could react. Furthermore, metabolic labeling showed that the putative novel class II-like TÛ39<sup>+</sup> non-DR, -DQ, -DP molecules actually were synthesized by the TCC themselves (Fig. 3*b*). In both surface-labeled and internally labeled cells, a relatively large amount of TÛ39-reactive material remained after depletion of the DR, DQ, and DP molecules, emphasizing the potential novel nature of these moieties. Their similarity to DR molecules may help to explain why they were not previously detected in two-dimensional gel analysis of whole lysates (20). The present results are consistent with the proposal that DY determinants, defined in functional assays, are carried by, or associated with, the putative novel TÛ39<sup>+</sup> non-DR, -DQ, -DP molecules demonstrated here. The TÛ39<sup>+</sup> novel class II-like molecules were, like the functionally defined DY determinants, also expressed on B-LCL (Table I). Metabolic labeling has confirmed that they were also synthesized by B-LCL (Fernandez, N., unpublished results). It will be necessary to confirm the novelty of DY by means of peptide mapping and amino acid sequencing, and to clarify the relationship between DY and established class II genes and products at the structural level.

DO $\beta$  remains a possible candidate class II gene for which a protein product has not yet been identified. Since it is differently regulated than other class II genes, it has been suggested that its function could also be different (9). It appears to be a gene of a relatively low degree of polymorphism, which is consistent with the findings pertaining to the DY LADs described here. However, DO $\beta$  mRNA has thus far not been found in T cells (9). DZ $\alpha$  (8) may also be a potential candidate for one of the DY chains, and this is known to be expressed in T cells (8). Another candidate may be the so-called "fourth Ia subset" described by Carra and Accolla (7), the nature of which is unknown, but which has also not yet been demonstrated in T cells. The retention of some LP stimulatory ability and antigen presentation capacity by class I and class II loss-deletion mutant B-LCL (36) also suggests the existence of functionally relevant non-DR, -DQ, or -DP molecules, which may therefore have some relationship with DY.

DY molecules clearly may not be the products of new class II genes, but rather

novel structures formed between  $\alpha$  and  $\beta$  chains of different known loci to generate mixed isotypes. The existence of such mixed isotype class II molecules has been demonstrated in mouse L cells transfected with murine class II genes (37). The relatively large amount of non-DR, -DQ, -DP T $\bar{U}$ 39<sup>+</sup> molecules on both B and T cells, and the failure thus far to isolate functional genes for class II molecules other than DR, DQ (and DX), DP, DO, and DZ, would be consistent with this possibility. Moreover, it has recently been reported that a variety of DR-, DQ-, DP-, and DO-containing mixed isotypes can be expressed after transfection of genes encoding the appropriate chains into L cells (11). Interestingly, these products were not detectable by DR-, DQ-, or DP-specific mAbs, but were, like DY, bound by broadly reactive class II-specific mAbs. Furthermore, the presence of such mixed isotypes has been implied, at least for DR $\alpha$ /DQ $\beta$  chains, in a more physiological system on B-LCL (12). Thus, a reasonable working hypothesis is that DY is a mixed isotype, or a group of mixed isotypes, expressed on B-LCL and on activated T cells other than helper cells.

A central role for DY LADs appears to be in the suppression of proliferative responses, since all PLT clones specific for DY were highly suppressive, and because only suppressive, but not otherwise equally class II-positive helper TCC expressed functionally defined DY on their surfaces. T $\bar{U}$ 39-inhibitable stimulation of LP responses in PBMC by suppressive clones is associated with induction of suppression (38), and, as shown here (Fig. 7), such stimulated PBMC can also respond to DY with limited autonomous proliferation. Autoreactive DY-responsive T cells were unable to help B cell Ig production (data not shown), and were not cytotoxic (16, Table IV). Thus far, the only effector function attributable to such cells is non-MHC-restricted potent suppression of LP responses stimulated in MLC (16, Fig. 5) and by mitogens and antigens (PHA and PPD, data not shown), and, like other DY<sup>+</sup> clones, the induction of SA in PBMC (Fig. 6). Since autoreactive clones both express and respond to DY, this would imply that the DY-associated suppressive circuit could be self maintaining by recruitment and by clonal expansion. However, the system presumably is kept in a steady state by the relatively meager quantities of IL-2 produced by the autoreactive SC, so that suppression would not always predominate, although a background level of suppressive activity would be present.

A degree of antigen specificity regulating the activity of the hypothesized DY-suppressor circuit would be mediated by the local availability of higher concentrations of IL-2 secreted by antigen-stimulated helper cells. This would be a simple strategy for upregulation of DY-stimulated suppression, which would then feed back to decrease helper function, thereby decreasing IL-2 availability, leading to its own downregulation. Further mechanisms for control of this type of suppression would undoubtedly also have to exist, possibly involving contra-suppression (39). Some preliminary evidence is consistent with this possibility (40).

### Summary

A set of T cell clones (TCC) isolated from HLA-DR-, Dw-, DQ-matched allogeneic MLCs was found to proliferate autonomously when stimulated with cells carrying a wide range of class I or II specificities. This apparently unre-

stricted proliferation was relatively weak, and only low levels of IL-2 were present in the supernatants of stimulated cells. Autologous as well as allogeneic PBMC and B lymphoblastoid cell lines (B-LCL) were capable of stimulating such clones, which were also restimulated by suppressive, but not by helper, TCC. Moreover, such clones displayed the unusual property of autostimulation. mAb inhibition experiments suggested that class II- or class II-restricted antigens were involved in stimulation. Thus, certain "broad" mAbs (TÜ39, SG520) reacting with multiple locus products inhibited activation of these reagents, but none of those reacting more specifically with DR (TÜ34, TÜ37, L243, Q2/70, SG157), DQ (TÜ22, SPV-L3, Leu10), or DP (B7/21), or mixtures of these mAbs, were able to do so. Evidence from sequential immunoprecipitation experiments suggested that mAb TÜ39 bound class II-like molecules other than DR, DQ, and DP on TCC and B-LCL, and it is therefore proposed that such putative novel class II-like molecules may carry the stimulating determinants for these autoreactive clones. DY-reactive clones lacked helper activity for B cells but mediated potent suppressive activity on T cell proliferative responses that was not restricted by the HLA type of the responding cells. Suppressive activity was induced in normal PBMC by such clones, as well as by independent suppressive clones, which was also inhibited only by mAb TÜ39. These findings lead to the proposal that DY-reactive autostimulatory cells may constitute a self-maintaining suppressive circuit, the level of activity of which would be regulated primarily by the availability of IL-2 in the microenvironment.

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