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Index to Volume 4

Abbas, A. K., see Mitchell, R. N. 189
Agostino, N., see Weber, D. A. et al. 255
Bach, F. H., see Houchins, J. P. et al. 295
Baluyut, A. R.: The Synergistic Effects of Anti-IgM and Monoclonal Anti-Ia Antibodies in Induction of Murine B Lymphocyte Activation 45
Barbis, D., see O'Garra, A. et al. 149
Baur, K., see Naparstek, Y. et al. 35
Bertoglio, J.: Expression and Regulation of Interleukin-1 mRNA and Interleukin-1 Receptors in Human B-Cell Lines 139
Bill, J.: Class I and Class II MHC Gene Products Differentially Affect the Fate of Vβ5 Bearing Thymocytes 269
Birkeland, M. L.: Epitopes on CD45R [T200] Molecules Define Differentiation Antigens on Murine B and T Lymphocytes 71
Björklund, M., see Forni, L. et al. 59
Braun, J.: Inducible Ig Heavy Chain Switching in an IgM^+ Ly-1 B Cell Line. Evidence for a State of Switch Commitment 105
Braun, J.: Unique V Gene Usage by B-Ly1 Cell Lines, and a Discordance between Isotype Switch Commitment and Variable Region Hypermutation 121
Breitman, L., see Naparstek, Y. et al. 35
Brocker, T., see Pawelec, G. et al. 21
Brooks, K. H.: Adrenocorticotropin (ACTH) Functions as a Late-Acting B Cell Growth Factor and Synergizes with Interleukin 5 327
Brooks, K. H.: Characterization of a Neoplastic B Cell Clone that Secretes IgM in Response to Th2-Derived Lymphokines 339
Buck, L. B., see Weber, D. A. et al. 255
Bühring, H. J., see Pawelec, G. et al. 21
Burn, P., see Kupfer, A. et al. 317
Busch, F. W., see Pawelec, G. et al. 21
Cernetti, C., see Ming, J. E. et al. 203
Chen, U., see Braun, J. et al. 105
Clark, M. E., see Braun, J. et al. 105
Coutinho, A., see Forni, L. et al. 59
Delohery, T. M., see Weber, D. A. et al. 255
Dileepan, K. N., see Yamada, A. et al. 191
Dosda, J., see Bertoglio, J. et al. 139
Evavold, B., see Quintans, J. et al. 225
Fernandez, N., see Pawelec, G. et al. 21
Fernandez-Botran, R., see Birkeland, M. L. et al. 71
Flood, P., see Ptak, W. et al. 281
Forni, L.: Membrane Expression of IgG but not Maturation to Secretion Requires DNA Replication 59
Fradelizi, D., see Bertoglio, J. et al. 139
Gibson, M.: A $CD5^+$ B Cell Hybridoma Derived Factor(s), which Induces Maturation of $CD5^+$, Idiotype-Specific B-Cell Populations 241
Govan, H. L., III, see Braun, J. et al. 105
Granelli-Piperno, A., see Ming, J. E. et al. 203
Harada, N., see O'Garra, A. et al. 149
Hardin, J. A., see Gibson, M. et al. 241
Hauptfeld, M.: Studies on Recombination within the Mouse *H-2* Complex: IV. Characterization of New Recombinant Haplotypes $H\text{-}2^{t7}$, $H\text{-}2^{t8}$, $H\text{-}2^{as2}$, and $H\text{-}2^{as3}$ 1
Hauptfeld, V., see Hauptfeld, M. et al. 1
Hirsch, R., see Quintans, J. et al. 225
Houchins, J. P.: Isolation and Characterization of NK Cell or NK/T Cell-Specific cDNA Clones... 295
Howard, M., see O'Garra, A. et al. 149
Janeway, C. A., Jr., see Portoles, P. et al. 129
Janeway, C. A., Jr., see Ptak, W. et al. 281

Kanagawa, O., see Bill, J. et al. 269
King, L., see Braun, J. 121
Krall, W. J., see Braun, J. et al. 105
Kupfer, A.: The PMA-Induced Specific Association of LFA-1 and Talin in Intact Cloned T Helper Cells 317
Lawton, A. R., see Rollins-Smith, L. A. 9
Lee, F., see O'Garra, A. et al. 149
Linten, J., see Bill, J. et al. 269
Loughnan, M. S.: A Regulatory Role for the Soluble IL-2 Receptor via Competition with the p75 Cell-Surface Form of the Receptor for IL-2 307
Madaio, M. P., see Naparstek, Y. et al. 35
Mak, T. W., see Naparstek, Y. et al. 35
Mayforth, R. D., see Quintans, J. et al. 225
McCann, J., see Noelle, R. J. 161
McSherry, C., see Houchins, J. P. et al. 295
Metlay, J., see Birkeland, M. L. et al. 71
Ming, J. E.: Interleukin 6 is the Principal Cytolytic T Lymphocyte Differentiation Factor for Thymocytes in Human Leukocyte Conditioned Medium 203
Mitchell, R. N.: A Commentary: Processing and Presentation of Antigens by B Lymphocytes 189
Miyokawa, N., see Houchins, J. P. et al. 295
Myers, C. D.: The Processing of Antigen and Anti-Ig by Antigen-Specific B Cells 179
Nagayama, A., see Soejima, T. et al. 87
Naparstek, Y.: Autoreactive T Cells with Atypical MHC Restriction from MRL-*lpr/lpr* Mice: Forbidden Clones Revisited 35
Noelle, R. J.: Cognate Interactions between Helper T Cells and B Cells. I. Cloning and Helper Activity of a Lymphokine-Dependent Helper T Cell Clone (Th-3) 161
Nossal, G. J. V., see Loughnan, M. S. 307
Oakley, C. S., see Brooks, K. H. et al. 339
O'Garra, A.: Constitutive Production of Lymphokines by Cloned Murine B-Cell Lymphomas—CH12 B Lymphoma Produces Interleukin-4 149
O'Neill, H. C.: Binding of Radiation Leukemia Viruses to a Thymic Lymphoma Involves Some Class I Molecules on the T Cell as Well as the T Cell Receptor Complex 213
Palmer, E., see Bill, J. et al. 269
Pawelec, G.: "Tolerization" of Human T-Helper Cell Clones by Chronic Exposure to Alloantigen: Culture Conditions Dictate Autocrine Proliferative Status but not Acquisition of Cytotoxic Potential and Suppressor-Induction Capacity 21
Pernis, B., see Weber, D. A. et al. 255
Portoles, P.: Asymmetry in the Recognition of Antigen: Self Class II MHC and Non-Self Class II MHC Molecules by the Same T-Cell Receptor 129
Ptak, W.: Antibody-Activated Immunoregulatory T Cells Are Defective in B Cell Deprived Mice .. 281
Puré, E., see Birkeland, M. L. et al. 71
Quintans, J.: B-Cell Transplants to Immunodeficient *xid* Neonates Do Not Imprint Their Helper T Cells 97
Quintans, J.: Direct Activation of Murine Resting T Cells by Con A or Anti-CD3 Ig 225
Reis, M. D., see Naparstek, Y. et al. 35
Rojo, J. M., see Portoles, P. et al. 129
Rollins-Smith, L. A.: Regulation of B-Cell Differentiation: Anti-μ Antibodies Have Opposite Effects on Differentiation Stimulated by Bacterial Lipopolysaccharide and 8-Mercaptoguanosine 9
Sado, T., see Soejima, T. et al. 87
Sanders, V. M., see Birkeland, M. L. et al. 71
Schneider, E. M., see Pawelec, G. et al. 21
Schwartz, R. S., see Naparstek, Y. et al. 35
Sherr, D. H., see Gibson, M. et al. 241
Shreffler, D. C., see Hauptfeld, M. et al. 1
Singer, S. J., see Kupfer, A. et al. 317
Soejima, T.: Acquisition of Repertoires of Suppressor T Cells under the Influence of Macrophages 87
Stancou, R., see Bertoglio, J. et al. 139

Stechschulte, D. J., see Yamada, A. et al. 191
Steinman, R. M., see Birkeland, M. L. et al. 71
Steinman, R. M., see Ming, J. E. et al. 203
Subbarao, B., see Baluyut, A. R. 45
Suzuki, T., see Yamada, A. et al. 191
Takayasu, H., see Brooks, K. H. et al. 339
Taniguchi, M., see Soejima, T. et al. 87
Trowbridge, I. S., see Birkeland, M. L. et al. 71
Utsunomiya, Y., see Bill, J. et al. 269
Vitetta, E. S., see Birkeland, M. L. et al. 71
Vitetta, E. S., see Myers, C. D. 179
Weber, D. A.: Class II MHC Molecules Are Spontaneously Internalized in Acidic Endosomes by Activated B Cells 255
Wemhoff, G. A., see Quintans, J. 97
Wernet, P., see Pawelec, G. et al. 21
Wollman, E., see Bertoglio, J. et al. 139
Yabe, T., see Houchins, J. P. et al. 295
Yamada, A.: Regulation of FCγ2a Receptor-Mediated Phagocytosis by a Murine Macrophage-Like Cell Line, P388D1: Involvement of Casein Kinase II Activity Associated with FCγ2a Receptor 191
Yokoyama, A., see Quintans, J. et al. 225
Zeff, R., see Hauptfeld, M. et al. 1

"Tolerization" of Human T-Helper Cell Clones by Chronic Exposure to Alloantigen: Culture Conditions Dictate Autocrine Proliferative Status but not Acquisition of Cytotoxic Potential and Suppressor-Induction Capacity

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Précis

Induction of clonal anergy in T-helper (Th) cells may have a role in regulating immune responses. A model system for studying Th cell tolerization at the clonal level *in vitro* could be useful for investigating the mechanisms involved. Accordingly, alloreactive helper cells were maintained in culture with interleukin 2 (IL 2) by intermittent stimulation with specific antigen. Regardless of the frequency of antigen stimulation, clones of age <ca. 35 population doublings (PD) were found to undergo antigen-specific autocrine clonal expansion in the absence of exogenous IL 2. Such young clones (designated as phase I) could therefore not be "tolerized" by frequent exposure to antigen. In contrast, most clones of age >ca. 35 PD could be tolerized by frequent exposure to antigen (designated as phase II clones). Their autocrine proliferation was then blocked, although they still recognized antigen specifically as shown by their retained ability to secrete interferon-gamma (IFN-γ) and granulocyte-macrophage colony stimulating factor (GM-CSF). The mechanism of response failure involved both an inability to upregulate IL 2 receptors in the absence of exogenous IL 2, as well as an inability to secrete IL 2. These defects were not overcome by stimulation with mitogens or calcium ionophore and phorbol esther in place of alloantigen. T-cell receptor α, β, and γ-chain gene rearrangements remained identical in phase I and phase II clones. Tolerization of phase II clones could be avoided by increasing the period between antigen exposures. Despite this, whether or not phase II cells were capable of autocrine proliferation, they were found to have acquired the novel function of inducing suppressive activity in fresh lymphocytes. Suppressor-induction was blocked by the broadly reactive MHC class II-specific monoclonal antibody (moAb) TÜ39, but not by moAb preferentially reacting only with HLA-DR, DQ, or DP. Sequential immunoprecipitation on T-cell clones showed the presence of a putative non-DR, DQ, DP, TÜ39^{+} molecule on phase II clones. However, this molecule was also found on phase I clones. The nature of the TÜ39-blockable suppressor-inducing determinant present on phase II but not on (most) phase I clones thus remains to be clarified. In addition to suppressor-induction activity, phase II clones also acquired lytic potential as measured in a lectin approximation system. Cytotoxic (CTX) potential was also not influenced by the frequency of antigenic stimulation and could be viewed as a constitutive modulation of clonal function.

These results demonstrate age-related alterations in the functional status of monoclonal Th cell populations in terms of their tolerizability for autocrine proliferation (which depends on the frequency of antigen stimulation in culture), and their acquisition of suppressor-inducer capacity and CTX potential (which appear to be constitutive properties of these clones).

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Similar modulations of function *in vivo,* should they occur, may be of importance in the establishment and maintenance of tolerant states.

Introduction

Normal human T lymphocytes cultured with stimulating antigen and interleukin 2 (IL 2) for extended periods have been reported to retain specificity and function [1–3]. However, numerous studies have explored alterations in functional status of T cell clones (TCC) during continued culture [1–7]. It has also been reported that "tolerization" of TCC may occur after treatment either with high doses of antigen and/or inappropriately presented antigen, which results in apparently permanent nonreactivity [8–10] or with high doses of IL 2, the effects of which may be temporary [11].

In the present article, we examine an alteration in the functional status of human alloreactive Th-TCC which appears to be a constitutive property of the clones, but which is superimposed on a second functional modulation dictated by culture conditions. We previously reported that the majority of Th-TCC lost their autocrine proliferative capacity after ca. 35 PD [12]. This appeared to be a permanently altered state because none was found to regain autocrine proliferation on continued culture with antigen and IL 2. Moreover, these TCC were found to have acquired suppressive activity for lymphocyte proliferative responses and the ability to induce suppressive activity in normal peripheral blood mononuclear cells (PBMC). Cell contact and proliferation of responding cells was required for the induction of suppressive activity and this could be inhibited by certain broadly reactive MHC class II-specific monoclonal antibodies (moAb) [12]. The induced suppressive cells were capable of limited autocrine proliferation and this stimulation was blocked only by the same broadly reactive class II moAb [13]. These stimulatory determinants were operationally designated "DY" [14].

The studies alluded to above were all performed with TCC which were propagated by weekly stimulation with specific alloantigen, and given crude IL 2 preparations two or three times per week. In order to investigate the reasons for modulation of function of alloreactive TCC under conditions of chronic antigenic stimulation, we cultured TCC with antigen and purified IL 2 preparations and examined the expression of T-cell antigen receptor (TCR) using moAb, as well as functional status in readout systems not involving clonal expansion (secretion of GM-CSF and IFN-γ), CTX and suppressor-inducer activity. In addition, alterations in TCR α, β, and γ chain gene rearrangement profiles using Southern blotting techniques were sought. The results reported here suggest that functional modulation within Th-TCC in the direction of suppressor-induction and CTX is a constitutive property of these aging cells, whereas autocrine clonal expansion is environmentally regulated by the availability of specific antigen during propagation.

Results and Discussion

Alterations in autocrine proliferative status of alloreactive TCC under different conditions of antigen exposure

Taking clones 7 d after stimulation and cryopreserved at different ages during their regular maintenance cycle (weekly antigen stimulation), it was confirmed that alloreactive phase I TCC had lost autocrine proliferative capacity by ca. 35 PD (Table 1). This correlated with a reduced ability to upregulate IL 2 receptors (IL 2R), as detected with moAb TÜ69, in the absence of exogenous IL 2 (Fig. 1). This was not a result of a shift in the kinetics of the response (data not shown). Even when such phase II cells were restimulated in the presence of exogenous IL 2, levels of ^{3}H-TdR incorporation were reduced compared to phase I cells (Table 1), and this was reflected in a slowdown of growth in maintenance culture for most clones (data not shown). However, by increasing the period between exposure to antigen from 5–7 d to 9–12 d, before testing autocrine proliferation, TCC were shown to retain proliferative status beyond 35 PD (Table 1). If maintained for longer than two weeks in the absence of antigen, the TCC died off. By restimulating with antigen every 10 d, autocrine proliferative capacity could be retained for the entire finite lifespan of the TCC (ca. 55–65 PD for most clones).

The defective response of phase II cells was further investigated using stimulating agents which bypassed activation signals mediated by the TCR-CD3 complex. It was found that phase II cells had also lost the ability to respond to phorbol esther (TPA) and calcium ionophore (ionomycin, Io). This is illustrated for clone 257-6 in Figure 2. Although phase I cells 5 d after antigen stimulation responded well to antigen alone or to 10 to 200 ng/ml TPA combined with 500 to 1000 ng/ml Io (but to neither agent alone) in the absence of antigen (data not shown), phase II cells 5 d after restimulation responded neither to antigen nor to combinations of TPA together with Io (Fig. 2). However, the same phase II cell culture 9 or 12 d after antigen stimulation regained autocrine proliferative capacity when stimulated by either antigen or TPA + Io.

Table 1. Autocrine proliferative responses of phase I and II Th-TCC at different times after antigen stimulation.

Clone		Medium	Medium + Stimulator[a] IL 2	Spec. stim.	Spec. Stim. + IL 2
248-3	Phase I	253 ± 101[b]	6728 ± 769	42744 ± 5108	67285 ± 5490
250-7	(<30PD)	148 ± 74	10334 ± 993	28935 ± 2604	55629 ± 6322
257-6	7 d after	632 ± 228	7335 ± 830	39831 ± 3807	70253 ± 7236
260-4	antigen	555 ± 238	5639 ± 879	46384 ± 4288	52385 ± 6000
248-3	Phase II	628 ± 242	3207 ± 1141	935 ± 307	4012 ± 938
250-7	(35–40PD)	527 ± 110	4529 ± 2440	833 ± 256	5630 ± 605
257-6	7 d after	1322 ± 345	2999 ± 349	1735 ± 143	7634 ± 962
260-4	antigen	400 ± 211	2566 ± 273	534 ± 138	3877 ± 633
248-3	Phase II	423 ± 42	3343 ± 590	39653 ± 4122	58354 ± 4834
250-7	(35–40PD)	356 ± 148	4547 ± 265	23042 ± 1924	36278 ± 4024
257-6	12 d after	893 ± 213	3345 ± 563	41562 ± 3908	54279 ± 6133
260-4	antigen	635 ± 264	2675 ± 632	59386 ± 5281	63900 ± 7072

[a] Stimulating agents were 20 U/ml IL 2 and/or 2.5 × 10^4/well specific alloantigen-positive irradiated B-LCL cells, on 1 × 10^4 TCC. Culture duration, 66 h; altering kinetics did not change pattern of results (data not shown).
[b] Data given as mean CPM of triplicate cultures ± SDM.

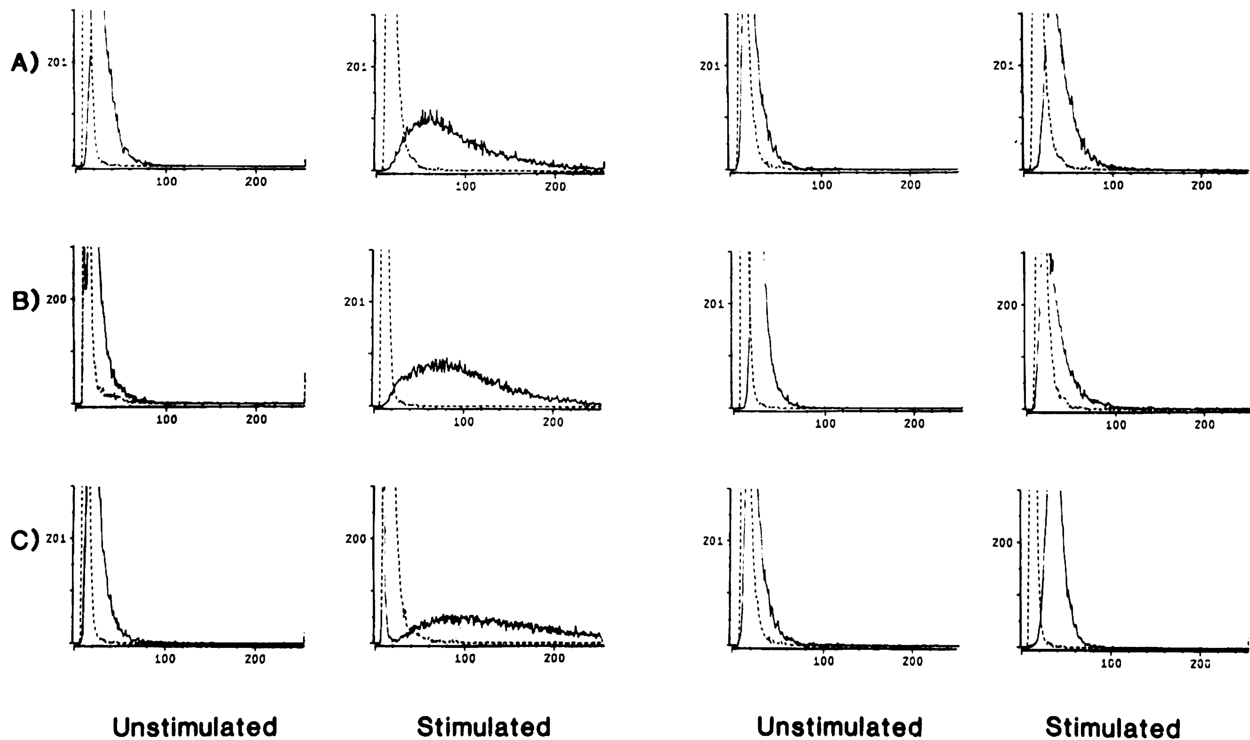

Fig. 1. Expression of IL 2R on Th-TCC after antigen stimulation. Th-TCC 248-3 (A), 250-7 (B) and 257-6, (C) were taken from maintenance culture 7 d after antigen stimulation (unstimulated), or rechallenged with specific KR-LCL for 48 h (stimulated) prior to assessing IL 2R expression using moAB TÜ69 in linear FACS. The left-hand side of the figure shows marked upregulation of IL 2R by phase I cells, whereas the right-hand side shows only marginal upregulation of IL 2R by phase II cells.

Lymphokine production by phase I and II TCC

As shown above, phase II clones 7 d after exposure to antigen were no longer capable of autocrine proliferation. This may have been due entirely to their inability to upregulate IL 2R in the absence of exogenous IL 2. However, it had previously been established that they were also defective in IL 2 secretion [12]. It was therefore asked whether secretion of lymphokines thought to be unrelated to the

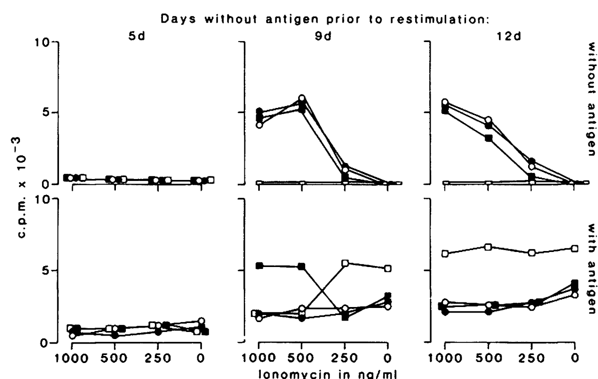

Fig. 2. Autocrine proliferative responses of phase II cells at various times after antigen stimulation. Phase II cells from clone 257-6 were cultured with specific alloantigen and IL 2 for 5, 9 and 12 d prior to harvest and stimulation by a variety of agents. The upper part of the figure shows responses to titrated doses of Io and TPA in the absence of specific antigen, while the bottom part of the figure shows the same experiment performed in the presence of specific alloantigen. Io was titrated as shown, TPA as follows: ●, 200; ○, 100; ■, 10; and □, 0 ng/ml.

proliferative requirements of the cloned cells themselves was coordinately downregulated or whether antigen-specific reactivity of phase II clones could still be demonstrated. For this purpose, secretion of GM-CSF and IFN-γ was studied. Factors supporting GM colony formation from normal human BM cells were found to be secreted in large amounts by phase I clones after specific stimulation but not after third-party stimulation (Table 2). Phase II clones retained this capacity, showing that antigen-specific effects were still measurable in these cells. Thus, the TCR-CD3 complex was still capable of delivering activation signals to the cells. A similar antigen-specific stimulation of GM-CSF secretion by phase I clones and its retention by phase II clones has also been observed in nine additional clones (data not shown). Furthermore, a similar result was obtained for the secretion of IFN-γ, another lymphokine thought not to be involved in the clonal expansion of the TCC themselves. Phase II cells retained the ability to secrete this lymphokine (Table 3). These results appear to bear some similarity to a recent murine model in which T cells may be "tolerized" by exposing them to antigen on 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide-treated antigen presenting cells. Such T cells failed to secrete IL 2 but retained their ability to secrete IFN-γ and IL 3 [15].

Modulation of function in Th-TCC: Cytotoxic status

The previous results suggested that phase II Th-TCC 5–7 d after antigen stimulation had retained functional, antigen-specific TCR but were unable to undergo autocrine clonal expansion due to a block at a stage distal to TCR-CD3-mediated signalling. It was therefore investigated whether other changes in their functional status had occurred in the phase II state. The CTX activity of these cells was tested since we had previously demonstrated acquisition of a weak MHC-unrestricted "NK-like" CTX by the majority of aged clones cultured with T cell-conditioned medium as a crude source of IL 2 [12]. Others had also reported the acquisition of allospecific CTX by TCC [16]. However, none of the present clones in phase I or phase II were CTX on either allospecific targets or on highly NK-susceptible K562 targets in a 4 h^{51}Cr-release assay (Table 4). Nonetheless, all the phase II, but not the phase I, cells mediated lectin (PHA)-dependent CTX on K562, suggesting that the acquisition of CTX machinery may be a general characteristic of phase II cells 7 d after antigen stimulation. Moreover, phase II cells 12 d after antigen stimulation, which had regained autocrine proliferative capacity, nonetheless retained their acquired lectin-dependent CTX (data not shown).

Modulation of function in Th-TCC: Suppressive activity and expression of "DY"

Phase II TCC were found to induce suppressive activity in normal PBMC. The induction of suppressive activity was blocked by moAb TÜ39 but not by TÜ22 (DQ), TÜ34 (DR), or B7/21 (DP). The TÜ39-inhibitable suppressor-inducer status of the TCC correlated with their expression of "DY"-determinants detected by primed lymphocyte typing [13]. Thus, only phase II, but not phase I, cells were functionally "DY"-positive, as shown by their stimulatory capacity on "DY"-reactive clones. Furthermore, Figure 3 shows that phase II cells which had regained autocrine proliferative capacity >9 d after antigen stimulation retained their acquired ability to induce suppressive activity. Thus, phase I TCC 5 d after antigen stimulation did not induce suppressive activity in normal PBMC, but phase II cells did induce suppression, regardless of whether they were tested 5, 9, or 12 d after antigen stimulation (Fig. 3). In these respects, acquisition of suppressor-inducer capacity and CTX appear to be preprogrammed events which occur independently of environmentally determined alterations in autocrine proliferative status.

MHC class II expression by phase I and II Th-TCC

The induction of suppression by phase II clones

Table 2. Specificity of stimulation for GM-CSF secretion by phase I and II Th-TCC

	248-3		250-7		257-6		260-4	
Stim.[a]	I	II	I	II	I	II	I	II
KR5	96 ± 5[b]	50 ± 3	74 ± 6	63 ± 4	93 ± 7	76 ± 3	75 ± 3	49 ± 4
WH5	94 ± 18[c]	95 ± 16	98 ± 15	95 ± 16	125 ± 17	92 ± 22	98 ± 11	96 ± 3
WK1	25 ± 6	12 ± 3	2 ± 1	9 ± 5	1 ± 1	0	3 ± 2	0
GM2	20 ± 5	1 ± 1	37 ± 3	0	13 ± 1	0	9 ± 3	4 ± 4
Medium	4 ± 1	0	12 ± 3	2 ± 1	0	0	5 ± 3	3 ± 2

[a] Stimulators (Stim.) were B-LCL from the donor of the original sensitising cells, KR5 (HLA-DR5), from WH5 (DR5), WK1 (DR1), or GM2 (DR2), or no stimulator (Medium).
[b] % of maximal colony formation supported by 10% HPCM (a plateau concentration in these experiments).
[c] % of colony formation ± SDM (mean of at least 3 experiments) supported by supernatant of Th-TCC stimulated with specific antigen (KR5 B-LCL). The results shown are for 10% Th-TCC supernatant harvested 48 h after stimulation. 80 Gy-irradiated B-LCL alone have not been observed to produce GM-CSF. Titrating these from 20–2% in initial experiments, or altering kinetics of production, did not significantly alter the pattern of the result.

Table 3. Secretion of IFN-γ by phase I and II Th-TCC

	250-7		257-6		260-4	
Stim.	I	II	I	II	I	II
KR5	>1000[a]	>1000	>1000	>1000	>1000	>1000
WH5	nt	420	nt	304	nt	728
WK1	nt	<15	nt	<15	nt	<15
GM2	nt	<15	nt	<15	nt	<15
Medium	nt	<15	nt	<15	nt	<15

[a] IFN-γ content in U/ml estimated from duplicate determinations by IRMA. TCC supernatants were harvested at 48 h after stimulation as in Table 2. Controls of B-LCL alone were consistently negative. The pattern of IFN-γ secretion at 96 h was essentially identical, although titers were commonly lower than at 48 h.

Table 4. CTX activity of phase I and II Th-TCC

Clone	Phase[a]	K562	K562 + PHA	Spec. LCL
248-3	I	<0.1	1.3	<0.1
	II	<0.1	523	<0.1
250-7	I	<0.1	0.9	<0.1
	II	3.8	738	0.5
257-6	I	<0.1	<0.1	<0.1
	II	1.1	489	6.9
260-4	I	0.4	3.9	<0.1
	II	5.6	872	3.7
290-2	NK-like[b]	972	4518	<0.1
281-17	CTL[c]	<0.1	1719	929

[a] Phase I and II Th-TCC were tested 7 d after antigen stimulation at titrated effector:target cell ratios, and data are given as lytic units (25% lysis) per 10^7 effector cells on 4000 targets.
[b] Clone 290-2 is a $CD3^+$ $CD4^{\pm}$ $CD8^-$ $Leu19^+$ cell which lyses only NK-susceptible target cells (Pawelec et al., manuscript submitted for publication).
[c] Clone 281-17 is a $CD3^+$ $CD4^-$ $CD8^+$ $Leu19^-$ allospecific CTX clone (Pawelec et al., unpublished results).

could be blocked by broadly reactive class II moAb TÜ39 but not by anti-DR, DQ, or DP moAb [12, 17]. Immunoprecipitation experiments in which DR, DQ, and DP molecules were sequentially depleted from lysates of surface ^{125}I labeled B- or T-cell lines demonstrated the presence of putative novel DR, DQ, DP^- $TÜ39^+$ class II-like molecules [18]. These were therefore considered to be candidates for the structures carrying functionally defined "DY"-suppressor-inducer determinants. Accordingly, phase I and II TCC were subjected to this type of analysis. Figure 4 shows that lysates of a phase II TCC do indeed contain such candidate "DY" molecules. However, the figure also shows that the same TCC studied in phase I contains this molecule as well. Preliminary quantification experiments on two clones suggest that the absolute quantity of "DY" candidate molecules on phase II cells is greater than on phase I cells (data not shown). If this is confirmed in further experiments, then a quantitative increase of "DY" molecules on phase II cells might explain their functional DY-positivity.

TCR gene rearrangements in phase I and II Th-TCC

DNA was isolated from phase I and phase II Th-TCC, and from subclones with or without autocrine proliferative capacity. The first experiments were limited to investigation of TCR β-chain (and γ-chain) gene rearrangements. The Southern blots clearly showed conservation of monoclonally rearranged bands in TCC and subclones, regardless of their age or functional status [13]. These results, which were obtained in all Th-TCC tested thus far (n = 6) are consistent with a lack of alteration of the TCR in phase II cells. Alterations in rearrange-

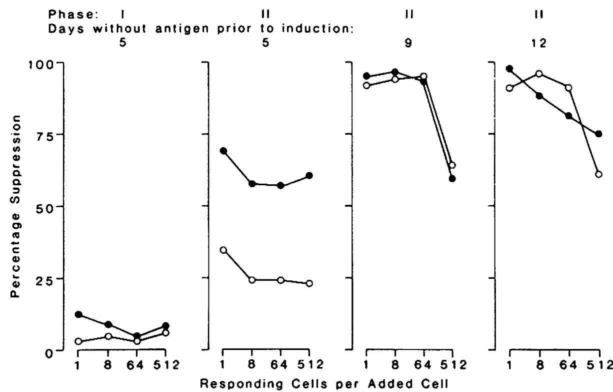

Fig. 3. Ability of phase II Th-TCC to induce suppressive activity. Th-TCC were irradiated and mixed 1:20 with normal PBMC and cultured for 3 d in medium containing human serum but no IL 2. These cells were then harvested, irradiated and titrated into MLC between the clone donor (●) or an allogeneic donor (○) and an irradiated PBMC pool, at the ratio shown (responding cells per added cell). This experiment was performed with cells from clone 257-6 in phase I, 5 d after antigen stimulation and in phase II, 5, 9, and 12 d after antigen stimulation, as shown.

ments of α chain genes in phase II was rendered unlikely by the finding that clone 260-4, which was the only informative clone of 14 tested, demonstrated the same rearranged band in phase II as in phase I (Fig. 5). In other respects this clone behaved identically to the remaining clones, including its retention of antigen-specific GM-CSF secretion capacity. Therefore, altered functions in phase II compared to phase I TCC are not associated with alterations in α-chain gene rearrangements.

Summary

Human Th clones maintained under conditions of chronic antigenic stimulation lost autocrine proliferative capacity and acquired lytic potential and suppressor-induction capacity. The former but not the latter changes could be modulated by culture conditions: namely, decreasing the frequency of exposure to antigen resulted in retention of autocrine proliferative capacity, but did not prevent the other functional alterations in the clones. Specific antigen recognition by the cells was, however, maintained in all functional states, as reflected by antigen stimulated GM-CSF and IFN-γ release. Aging cloned cells therefore appear to be committed to modifying their immunomodulatory activity while maintaining their antigenic specificity.

Interpretation

A loss of allostimulated autocrine proliferative capacity occurring after extended culture of human helper T-cell clones has previously been reported by ourselves [12] and others [7, 19, 20]. We noted that the 90% of alloreactive PLT clones which lost autocrine proliferative capacity after a period in culture acquired suppressive activity for responses in MLC [12, 13]. Suppression was associated with an acquired suppressor-inducer function which could be inhibited by the MHC class II-specific moAb TÜ39 [12], or by the IL 2R-specific moAb TÜ69 [17]. This suggested that cell:cell contact involving MHC products, and IL 2-driven proliferation of the stimulated cells was necessary for the induction of suppressive activity. Suppression was not caused by IL 2 absorption, and as far as could be established, was not associated with de novo contamination with mycoplasma or acquired nonspecific CTX [12].

Such modulations in the function of Th-TCC are consistent with the idea that a strict regulation of helper activity exists even within monoclonal populations, as has been previously hypothesised [21]. Limiting dilution and subcloning experiments which showed that the frequency of proliferative cells within monoclonal populations decreased with age, support this conclusion by suggesting the generation of nonreactive variants [13]. However, lack of autocrine proliferation by phase II cells was not simply due to the presence of a lower frequency of potentially reactive cells, since an extended restimulation kinetic did not allow a response to be visualized. Such phase II cells also failed to respond to stimulating agents (TPA + Io) which bypass TCR-CD3 signalling (Fig. 2). Failure to proliferate was caused by a reduced ability to upregulate IL 2R after stimulation in the absence of exogenous IL 2 (Fig. 1), as well as an inability to secrete IL 2 [12].

This loss of autocrine proliferative capacity in Th-TCC could be manipulated by altering culture conditions. When clones were cultured for 9–12 d without antigen stimulation, proliferative ability was retained until almost the end of the lifespan of the clones. This presumably reflects a more extended refractory period following antigen stimulation for phase II compared to phase I cells. The nature of this transient post-recognition block to proliferation is presently unknown. Fluctuations in the level of expression of TCR-CD3 complexes did not seem responsible because a) phase II cells expressed the same density of CD3 and WT31-reactive antigens as phase I cells [13], and b) stimulating agents which bypassed the CD3-TCR complex also failed to stimulate phase II cells which had not been "starved" of antigen. That this block was indeed post-recognitive was shown by retention of antigen-specifically stimulated release of GM-CSF

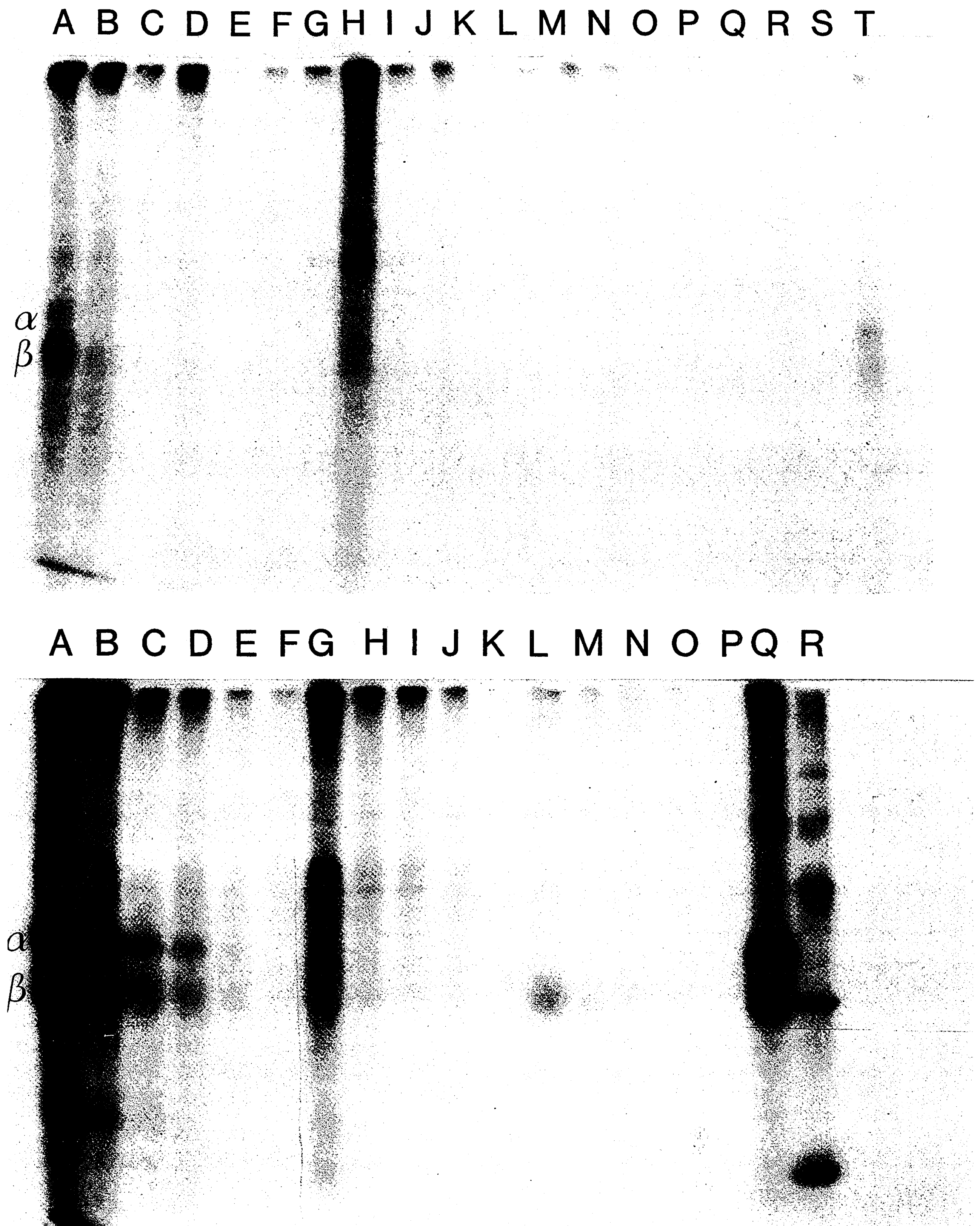

Fig. 4. Expression of "DY" molecules by phase I and II Th-TCC. Two million cell equivalents of ^{125}I-lactoperoxidase surface labeled cells from Th clone 257-12 in phase I (upper panel) or phase II (lower panel) were subjected to sequential immunoprecipitation with class II-specific moAb followed by SDS-PAGE: (upper panel) L243 (DR), lanes A–E and SACI-RAM alone lanes F, G: TÜ22 (DQ) lanes H–K and SACI-RAM alone lanes L, M; B7/21 (DP) lanes N–Q and SACI-RAM alone lanes R, S; and finally, TÜ39 ("DY"), lane T; (lower panel) L243 (DR), lanes A–D and SACI-RAM alone lanes E, F; TÜ22 (DQ) lanes G–I and SACI-RAM alone lanes J,K; B7/21 (DP), lanes L–N and SACI-RAM alone lanes O, P; and finally, TÜ39 ("DY"), lane Q. Lane R shows molecular weight markers.

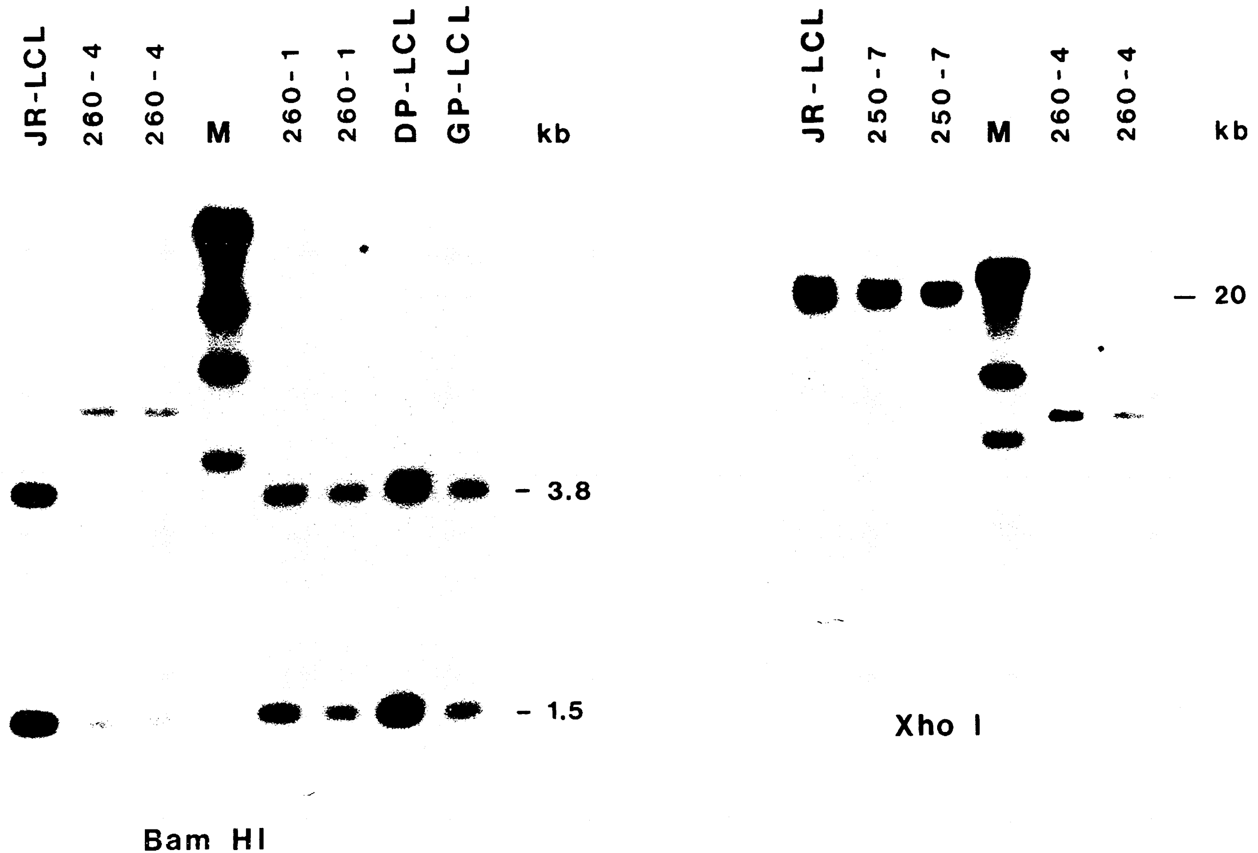

Fig. 5. Rearrangement of TCR α chain genes in phase I and II Th-TCC. DNA of Th-TCC in phase I and II was digested with Bam HI or Xho I as shown, and blotted with genomic TCR α chain probe D14S7c. Germ-line fragments of 3.5 and 1.8 kb for Bam HI and 20 kb for Xho I are shown from B-LCL (JR-, GP-, and DB-LCL). Sizes of restriction fragments were determined by co-electrophoresis of Hind III-digested phage-γ DNA (lane M), fragments of which were labelled by nick-translation and hybridised on the same filter.

and IFN-γ, implying that the TCR complex still transduced positive signals but that lymphokine secretion was noncoordinately regulated.

In contrast to the above situation for autocrine proliferation, phase II cells acquired suppressor-inducer capacity and lectin-dependent CTX activity apparently independently of environmental influences. In general, Th-TCC began to show suppressor-inducer activity concordantly with their loss of autocrine proliferative capacity [12]. Suppressive activity was positively correlated with the presence of lymphocyte stimulatory determinants designated "DY" [14, 22] on these clones [13]. Thus, nonsuppressive phase I clones and subclones were unable to stimulate "DY"-specific cellular reagents, but suppressive clones and subclones were able to do so [13]. This stimulatory capacity applied regardless of their proliferative status, since although antigen "starvation" allowed retention of autocrine proliferative capacity for extended periods, the acquisition of "DY" determinants was not affected by this treatment. Similarly, the acquisition of lectin-dependent CTX was age-related but not influenced by frequency of exposure to antigen.

The molecular nature of "DY" is under investigation. Sequential immunoprecipitation experiments suggest the existence of distinct TÜ39^{+} class II-like entities which are not precipitated by anti-DR, DQ, or DP moAb [18]. Although these are present on phase II Th-TCC they are, however, also detectable on phase I TCC (Fig. 4). Several possible explanations may account for this observation: 1) these extra molecules do not carry "DY", 2) of several extra molecules, only one carries "DY" and this is indeed lacking on phase I cells, 3) the extra molecules act as restriction elements for the presentation of peptides which are only available on phase II cells, 4) quantitative dif-

ferences in expression of extra molecules explain the functionally defined "DY"-positivity of phase II cells but "DY"-negativity of phase I cells. Further experimentation is required to distinguish between these, or other, possibilities.

The mechanism of suppression has not yet been resolved. It does not appear to involve CTX, although selective killing of stimulating cells such as dendritic cells cannot be formally excluded on the basis of the ^{51}Cr-release test. Acquisition of specific CTX was not observed in the present clones, although alloreactive and influenza virus-specific Th-TCC have been reported to lyse such targets after aging in culture or treatment with high concentrations of IFN-α [16, 23]. The latter was reported to be accompanied by alterations in the rearrangement of TCR α chain genes [23]. Since the single TCC informative for the α chain which we studied had not acquired alloCTX activity, our failure to find alterations in α chain gene rearrangements may still be compatible with this. The reason that only germline α-chain fragments were found with the D14S7c genomic α-chain probe in 13/14 Th-TCC was probably because their rearrangements involved J region segments upstream of the Bam HI and Xho I cleavage sites.

There may conceivably be circumstances under which an extended clonal expansion of helper cells, such as employed here *in vitro,* would result in the same kind of functional modulation *in vivo*. In chronic infections, after repeated blood transfusions or after organ transplantation, the continued stimulation of Th cells resulting in functional modulation could contribute to immunodeficiency. A report on defective T-cell reactivity in lepromatous leprosy is consistent with this concept [24]. Similarly the finding that, even under therapeutic immunosuppression, lymphocyte stimulation and generation of memory cells may still occur [25, 26] suggests similar mechanisms also in organ transplantation. After allogeneic bone marrow transplantation, T-cell proliferative responses remain depressed for an extended period after apparently successful reconstitution. This refractoriness is reflected in poor ability to upregulate the IL 2R and in mediocre IL 2 secretory capacity, although TCR function appeared normal as measured by Ca^{2+} ion influx [27]. Moreover, in a rat heart allograft model, it was recently reported that $CD4^+$ cells which underwent priming in the presence of CsA, lost their capacity to transfer rejection capacity, but rather became suppressive [28]. This modulation of function was dependent on the chronic presence of antigen, since alloreactivity was regained 8 d after removing the graft [29]. Under such conditions of chronic antigen stimulation, modulation of Th-cell function in the direction of suppression might occur in the majority of reactive clones.

Taken together, such possible *in vivo* parallels to phase I–phase II functional modulations could contribute to the establishment and maintenance of tolerant or immunodepressed states.

Material and Methods

T lymphocyte clones

The TCC studied here were derived from different donors sensitized in mixed lymphocyte cultures (MLC) to the same stimulating cells (homozygous typing cell KRO, 9W505, HLA genotype A2, B44, DRw11, Dw5, DRw52, DQw3, DPw2,4) by limiting dilution of alloactivated lymphocyte populations in the presence of IL 2 and stimulator-specific filler cells. Cultures with >75% lymphoblasts were selected for cloning, cells were diluted in RPMI 1640 + 16% HS supplemented with 20 U/ml highly purified natural IL 2 (Lymphocult T-HP, Biotest Serum Institute, Frankfurt, FRG), and seeded at limiting dilution (<0.45 cells/well) into 1 mm-diameter culture wells of microtest plates (Greiner, Nürtingen), with 1×10^4 30 Gy-irradiated stimulator-specific PBMC in a total volume of 20 µl. In addition to 300 wells seeded with <0.45 cells, 60 wells each at ×10 and ×100 this concentration were also set up to confirm single hit characteristics of the limiting dilution titration for each experiment (r = 1.0 in the present experiments). Contents of positive wells were transferred after 6–9 d to 7 mm-diameter flat bottom microtiter plate wells (Greiner, Nürtingen, FRG) in 200 µl fresh medium with 1×10^5 stimulator PBMC, and then again transferred to 16 mm-diameter cluster plate wells (Greiner, Nürtingen, FRG) with 2×10^5 stimulator PBMC. After 4 d, wells were split 1:1, adding fresh medium. After 7 d, cultures were split to new wells at 2×10^5/well with 1×10^5 80 Gy-irradiated B-LCL derived from the donor of the original stimulating cells. Thereafter clones were propagated by feeding with fresh medium every 3–4 d and restimulating with B-LCL every 7 d.

The age of the clones at the time of cryopreservation was estimated as the number of PD undergone. Calculations for further culture of thawed cells resumed at the point at which the cells had been frozen. Spot tests on culturing TCC for a week in the absence of antibiotics showed that none of the clones used had latent bacterial contamination. Mycoplasma testing was kindly performed using 4-wk agar cultures by the Hygiene Institute of the

University of Tübingen. None of the clones in the present study showed any signs of mycoplasma contamination.

The clones studied here were found in initial screening assays to proliferate strongly when challenged with KR cells in the absence of IL 2 (stimulation index >100). Their alloantigenic specificity was defined as HLA-Dw5-associated in population studies, family segregation analyses, and moAb inhibition experiments (data not shown). The clones were identified as helper cells on the basis of their ability to stimulate Ig production from enriched B cell populations (F. Falcioni, unpublished results).

Assay of proliferative responses

Autocrine proliferative responses were measured by plating TCC (usually 1×10^4) in U-well microtiter plates (Greiner, Nürtingen, FRG) with irradiated PBMC or LCL (usually 1×10^5 or 2.5×10^4, respectively) from HLA-typed donors in RPMI 1640 + 10% HS. In other experiments, TCC were stimulated by the phorbol esther tetradecanoyl-phorbol-13-acetate (TPA, Sigma, Munich, FRG) and the calcium ionophore ionomycin (Io, Calbiochem, San Diego, CA, USA) at titrated doses in the presence or absence of alloantigen. Incubation time was generally 42 or 66 h, including an 18-h pulse with 37 kBq/well tritiated thymidine (^{3}H-TdR, Amersham-Buchler, Braunschweig, FRG, specific activity 185 GBq/mmol). Incorporated radioactivity was quantified using β-scintillation spectroscopy. In some restimulation experiments, the medium was supplemented with 20 U/ml HP IL 2 or recombinant IL 2 (the latter a kind gift of Dr M. Wrann, Sandoz, Vienna, lot W3/6, activity ca. 4×10^6 units/mg protein). PHA (Gibco M, Scotland) was used at 1% final concentration.

Induction and measurement of suppression of lymphocyte proliferative responses

To test for suppressor-inducer capacity, irradiated TCC were cocultured for 3 days with normal PBMC at a ratio of 1:20 in RPMI 1640 + 10% human serum and 60 μg/ml tylosine (anti-PPLO agent, Gibco M, Scotland). These were then washed, irradiated, and titrated into readout MLC as previously described [12]. Briefly, MLC were set up between 5×10^4 responding PBMC and the same number of stimulating 20 Gy-irradiated PBMC or 30 Gy-irradiated pooled PBMC from 20 donors, in U-well microtiter plates. Cells to be tested for suppressive activity were irradiated 20 Gy and titrated into the readout MLC, usually starting at a ratio of 1:1. Culture kinetic was routinely 138 h, including the 18-h pulse with ^{3}H-TdR prior to termination. Results were expressed as a relative response in the presence of added cloned cells compared to the MLC value in the absence of added cells.

Assay of cytotoxicity

Standard 4-h ^{51}Cr-release assays were performed by coculturing labeled target cells (4000/well) with titrated numbers of TCC as effector cells. $2\text{–}3 \times 10^6$ target cells (K562 and B-LCL) were labeled with 9 MBq sodium ^{51}chromate (specific activity 22.2 MBq/mg chromium, Amersham-Buchler) for 1.5 h, and the isotope release assay was of 4 h duration. PHA was used at a concentration of 1%. In each experiment, positive controls for natural killer (NK) and allospecific CTX were included. Results were expressed as lytic units defined as the number of effector cells per 10^7 required to achieve 25% specific ^{51}Cr release from 4000 target cells.

Expression of IL 2R

T-cell clones labelled with moAb were developed with a FITC-conjugated F(ab)$_2$ rabbit anti-mouse Ig (Dianova) as the second antibody, and analyzed on a FACS IV. Dead cells exhibited a smaller forward light scatter intensity clearly separable from viable cells and were excluded from the analysis by electronic gating. The fluorescence histograms were area-corrected to 10,000 cells. To indicate background staining, histograms of cells labeled with test moAb are superimposed on those labeled with the nonbinding control moAb W6/32.HK. The IL 2R moAb employed was TÜ69.

Lymphokine and colony stimulating factor production and assay

4×10^5 rested cloned cells were restimulated in 16 mm-diameter culture wells with 5×10^5 B-LCL from HLA-typed donors for different periods of time before harvesting cell-free supernatants for assay. For quantification of IL 2 production, peak lymphokine release kinetics of 24 h applied, and IL 2 was detected by titrating supernatants onto IL 2-dependent T-cell lines. For studies of GM-CSF and IFN-γ production, clones were cultured with 10 U/ml IL 2 as well as B-LCL. For all lymphokine assays, controls consisted of cloned cells or stimu-

lator cells incubated in medium alone. IFN-γ was measured using an IRMA kit according to the manufacturer's instructions (Celltech, Slough, UK). Factors in supernatants of TCC which facilitated the growth of human bone marrow GM colonies were assayed in the absence of the standard source of GM-CSF (human placenta conditioned medium, HPCM). BM mononuclear cells (isolated on 1.077 g/cm^3 Percoll gradients) were cultured in a single layer methylcellulose system. 1×10^5 cells/ml were plated in 0.9% methylcellulose supplemented with 20% prescreened fetal calf serum and 10% v/v cloned cell-derived or control supernatant. Triplicate cultures were plated in 16 mm-diameter cluster wells, and colonies (>50 cells) were enumerated on d 14. Initial experiments established that peak GM-CSF occurred at 24–48 h. Titration experiments showed that plateau (HPCM) level colony formation was achieved using 10% Th-TCC supernatant.

Sequential immunodepletion procedures

For immunoprecipitation studies, moAb L243, TU22, B7/21, and TU39 were purified by Protein A Sepharose 4B affinity chromatography according to Ey et al. [30].

TCC were surface labeled with ^{125}I by the lactoperoxidase catalyzed method [31]. Briefly, 2×10^7 TCC were resuspended in 0.5 ml of cold saline to which 18,000 kBq of carrier-free sodium ^{125}I-iodate (Amersham) was added. In rapid sequence, 30 U of lactoperoxidase (Calbiochem, specific activity 90 U/ml) 15 μl of glucose oxidase (Sigma) and 75 μl of glucose (Sigma, 50 mg/ml) were added, and the reaction was stopped after 30 min by repeated washing in cold saline.

Immunoprecipitation studies were performed as previously described [32]. Briefly, labelled cell extracts were "precleared" with 100 μl of protein A-bearing Staphylococcus aureus Cowan I (SACI) and 50 μl of SACI coupled to rabbit anti-mouse globulin (SACI-RAM). Two million cell-equivalents were then incubated with 1 μg of Protein A Sepharose-purified moAb at 4° for 1 h. Immunocomplexes were then incubated with 50 μl SACI-RAM to ensure identical binding capacity of the SACI to the various moAb. The precipitates were then washed ×3 in lysis buffer without detergent. For sequential immunoprecipitations, the supernatant was retained for serial transfer at each step to tubes containing identical amounts (1 μg) of the different mAb, 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 [33] followed by fixing and fluorography in Amplify (Amersham) and autoradiography on Hyperfilm MP (Amersham) at −70°.

T-cell antigen receptor gene rearrangements

DNA from TCC was isolated according to standard procedures involving SDS-lysis, proteinase K digestion, phenol and chloroform extraction, followed by ethanol precipitation. Approximately 5 μg DNA of each sample were digested with the appropriate restriction enzyme at 5 units/μg DNA, according to the instructions of the manufacturer (Boehringer, Mannheim, FRG). Digested DNA was electrophoresed through 0.6% or 0.7% agarose gels, denatured by alkali, neutralized and transferred to nylon filters (Amersham). The TCR α chain genomic probe was a 1.5 kb Hind III fragment, designated D14S7C, of the clone K40 derived from the breakpoint region of a (11/14) translocation leukemia [34]. This was a kind gift of Dr. D. Mathieu-Mahoul, Paris, and was subcloned in the pSP4 vector. The probe was labeled by incorporating ^{32}PdCTP (1.1×10^{14} Bq/mmol, Amersham) using random oligonucleotide primers (Pharmacia, Freiburg, FRG) and the large fragment of DNA polymerase I (Klenow enzyme, Boehringer, Mannheim, FRG). The specific activity of the probe was approximately 1×10^9 cpm/μg of insert DNA. Filters were hybridized in 5 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na-citrate) at 65° for 12–16 h at a radioactive concentration of 5×10^6 to 1×10^7 cpm/ml. Final washing of the filters was in 0.1 × SCC & 0.1% SDS at 60–65°, which were then autoradiographed for 18–36 h at −70° using intensifying screens.

Acknowledgments: The assistance of Mss. A. Rehbein, I. Balko, K. Katrilaka, and C. Schmidt, and artwork by Ms. M. Dürnay is gratefully acknowledged. We thank Mr. F. Kalthoff for performing the Southern blotting experiments.

References

1. H Von Boehmer, W Haas (1985): T cell clones. Elsevier, Amsterdam.
2. CG Fathman, FW Fitch (1982): Isolation, characterisation and utilisation of T lymphocyte clones. Academic Press, Orlando.
3. M Feldmann, JR Lamb, JN Woody (1985): Human T cell clones. Humana Press, Clifton, NJ.
4. CG Brooks, DL Urdal, CS Henney (1983): Lymphokine-driven "differentiation" of cytotoxic T cell clones into cells

with NK-like specificity: correlations with display of membrane macromolecules. *Immunol. Rev. 72*:43.

5. MM Simon, HU Weltzien, HJ Bühring, K Eichmann (1984): Aged murine killer T-cell clones acquire specific cytotoxicity for P815 mastocytoma cells. *Nature 308*:367.
6. DB Wilde, FW Fitch (1984): Antigen-reactive cloned helper T cells. I. Unresponsiveness to antigenic restimulation develops after stimulation of cloned helper T cells. *J. Immunol. 132*:1632.
7. B Malissen, D Charmot, C Mawas (1981): Expansion of human lymphocyte populations expressing specific immune reactivities. III. Specific colonies, either cytotoxic or proliferative, obtained from a population of responder cells primed in vitro. Preliminary immunogenetic analysis. *Hum. Immunol. 2*:1.
8. JD Levich, DE Parks, WO Weigle (1985): Tolerance induction in antigen-specific helper T cell clones and lines in vitro. *J. Immunol. 135*:873.
9. JR Lamb, BJ Skidmore, N Green, JM Chiller, M Feldmann (1983): Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J. Exp. Med. 157*:1434.
10. MK Jenkins, RH Schwartz (1987): Antigen presentation by chemically-modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med. 165*:302.
11. DB Wilde, MB Prystowsky, JM Ely, SN Vogel, DP Dialynas, FW Fitch (1984): Antigen-reactive cloned helper T cells. II. Exposure of murine cloned helper T cells to IL 2-containing supernatant induces unresponsiveness to antigenic restimulation and inhibits lymphokine production after antigenic stimulation. *J. Immunol. 133*:636.
12. G Pawelec, EM Schneider, P Wernet (1986): Acquisition of suppressive activity and natural killer-like cytotoxicity by human alloproliferative "helper" T cell clones. *J. Immunol. 136*:402.
13. P Wernet, EM Schneider, F Kalthoff, G Pawelec (1987): Constitutive functional heterogeneity without detectable somatic mutation of antigen receptor genes in helper T cell clones: Possible regulation by novel HLA class II "DY" determinants. *Immunol. Rev. 96*:109.
14. P Wernet, G Pawelec, EM Schneider (1986): Cellular detection of human class II antigens: Delineation of a novel HLA-DP-like suppressor restriction system, DY, the sequential expression of class II antigens, and a pronounced functional flexibility of class II alloproliferative T cell clones. In: HLA class II antigens. A comprehensive review of structure and function. B. G. Solheim, E. Moller, and S. Ferrone, eds. Springer-Verlag, Heidelberg, p. 281–298.
15. MK Jenkins, DM Pardoll, J Mizuguchi, TM Chused, RH Schwartz (1987): Molecular events in the induction of a nonresponsive state in IL 2-producing helper T lymphocyte clones. *Proc. Natl. Acad. Sci. USA 84*:5409.
16. B Fleischer (1984): Acquisition of specific cytotoxic activity by human T4$^+$ T lymphocytes in culture. *Nature 308*:365.
17. G Pawelec, EM Schneider, A Rehbein, I Balko, P Wernet (1986): Dissection of suppressor cell generation in vitro. *Hum. Immunol. 17*:343.
18. G Pawelec, N Fernandez, T Brocker, EM Schneider, H Festenstein, P Wernet (1988): "DY"-determinants, possibly associated with novel class II molecules stimulate autoreactive CD4$^+$ T cells with suppressive activity. *J. Exp. Med. 167*:243.
19. R Duquesnoy, A Zeevi (1983): Immunogenetic analysis of the HLA complex with alloreactive T cell clones. *Hum. Immunol. 8*:17.
20. NR Reinsmoen, A Anichini, FH Bach (1983): Clonal analysis of T lymphocyte response to an isolated class I disparity. *Hum. Immunol. 8*:195.
21. G Pawelec, EM Schneider, P Wernet (1983): Human T cell clones with multiple and changing functions: indications of unexpected flexibility in immune response networks? *Immunol. Today 4*:275.
22. G Pawelec, P Wernet, R Rosenlund, M Blaurock, EM Schneider (1984): Strong lymphoproliferative suppressive function of PLT clones specific for SB-like antigens. *Hum. Immunol. 9*:145.
23. L-K Chen, D Mathieu-Mahul, FH Bach, J Dausset, A Bensussan, M Sasportes (1986): Recombinant interferon-alpha can induce rearrangement of T cell antigen receptor alpha-chain genes and maturation to cytotoxicity in T lymphocyte clones in vitro. *Proc. Nat. Acad. Sci. USA 83*:4887.
24. N Mohagheghpour, RR Gelber, EG Engleman (1987): T cell defect in lepromatous leprosy is reversible in vitro in the absence of exogenous growth factors. *J. Immunol. 138*:570.
25. PM Chisholm, MT Drayson, JH Cox, WL Ford (1985): The effects of cyclosporin on lymphocyte activation in a systemic graft-vs.-host reaction. *Eur. J. Immunol. 15*:1054.
26. G Milon, P Truffa-Bachi, B Shidani, G Marchal (1984): Cyclosporin A inhibits the delayed-type hypersensitivity effector function of T lymphocytes without affecting their clonal expansion. *Ann. Immunol. 135D*:237.
27. M Lopez-Botet, MO de Landazuri, M Izquierido, A Ramirez, A Figuera, R Camara, J Fernandez-Ranada (1987): Defective interleukin 2 receptor expression is associated with the T cell dysfunction subsequent to bone marrow transplantation. *Eur. J. Immunol. 17*:1167.
28. BM Hall, KE Gurley, SE Dorsch (1987): Tempo of induction of W3/25-positive suppressor cells in cyclosporine-treated rat cardiac allograft recipients. *Transplant. Proc. 19*:504.
29. BM Hall, K Gurley, SE Dorsch (1987): Specific unresponsiveness in rats with prolonged allograft survival is dependent upon the graft and suppressor T cells. *Transplant. Proc. 19*:495.
30. PL Ey, SJ Prowse, CR Jenskin (1978): Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum protein using Protein A-sepharose. *Immunochem. 15*:429.
31. A Hubbard, Z Cohn (1972): The enzymatic iodination of the red cell membrane. *J. Cell. Biol. 55*:390.
32. N Fernandez, M Labeta, M Kurpisz, R Solana, H Festenstein (1986): HLA-DQ molecular heterogeneity in HLA-DR4-Dw4 consanguineous cell lines. *Disease Markers 4*:191.
33. UK Laemmli (1970): Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature 227*:68.
34. D Mathieu-Mahul, JF Caubet, A Bernheim, M Mauchaffe, E Palmer, R Berger, C-J Larsen (1985): Molecular cloning of a DNA fragment from human chromosome 14 (14q11) involved in T cell malignancies. *EMBO J. 4*:3427.

A Critique

This paper covers two distinct topics relating to long-term growth of alloreactive CD4 (''helper'') T cell clones. First, that after time, the clones require a rest after antigenic restimulation before they can again respond to alloantigen by proliferation. Second, that after time, all such clones develop non-specific cytotoxicity (but *not* specific cytotoxicity), and also suppress MLR.

Questions one would like to have answered are:

1. Does the nature of the stimulus determine acquisition of phenotype? The authors use LCL's, while in the mouse spleen cells are used, and are necessary to maintain growth and specificity. This is an obvious difference.
2. Does ECDI treatment, or ionomycin treatment, generate ''Phase II'' responses of ''Phase I'' lines?
3. What does change about phase II cells to account for the results? Is the chemistry of the receptor distinct.
4. What are DY antigens? This is a whole separate story and should be removed to focus on the ''tolerance'' issue.

Charles A. Janeway, Jr.

A Critique

The manuscript describes experiments on human T helper cell clones and their longterm behavior *in vitro*. During the course of long-term culture, the cells become dependent on Interleukin-2, and acquire suppressive and cytotoxic function. It has been observed, that the frequency of antigen restimulation influences the time point at which the clones become dependent on Interleukin-2. In contrast, the cells acquire suppressive and cytotoxic activities at time points independent of the frequency of antigenic restimulation.

The manuscript describes a careful and detailed study on the behavior of the cell lines, including cellular and molecular analyses. The study is of interest for those who work with long-term *in vitro* cultures of human T lymphocytes.

The experiments do not address the point of T helper cell tolerance, which the authors claim in the précis and at various points in the text. It is very unlikely that T cell undergoes 35 duplications *in vivo*, the time point at which in this paper a frequently antigen exposed T cell clone becomes ''tolerized.''

The Authors Respond

Response to first critique

Point 1) we have found essentially similar behaviour on the part of clones cultured on peripheral blood cells rather than B cell lines (this is documented in Ref. [12] of the article, *JI 136*:402, 1986).

Point 2) it is not possible to maintain clonal expansion with phorbol esthers and/or calcium ionophores, so this question cannot be answered.

Point 3) we do not have any data on the chemistry of the antigen receptor on these clones, but have shown in the present manuscript that it continues to function in an antigen-specific manner. I think that this makes it unlikely that modifications to the receptor itself play a part. Other changes in phase II cells concern increased density of class II molecules including the ''DY''-candidate molecule (see Fig. 4) which we argue may be related to one of the functional changes observed, ie. induction of suppressive cells by ''DY''-positive clones.

Point 4) ''DY''-determinants are an integral part of the ''active'' tolerance story, are not a separate story and should not be removed from the paper.

Response to second critique

The Reviewer believes that our use of the term ''tolerance'' is inappropriate, arguing that it would be unlikely that a T cell would undergo 35 population doublings before the phenomena which we

have described *in vitro* would occur *in vivo*. However, this is purely a matter of opinion, since no data on this topic are extant. We would argue that at least under certain conditions, for example in chronic infections or in allograft rejection episodes, it is easily conceivable that this number of doublings could take place. We would like to take this opportunity further to clarify our use of the term "tolerization." In the present manuscript, we address the question of tolerance in an *in vitro* model at two distinct levels, as implied by the title of the communication; namely that which could be designated "passive" and that which could be designated "active." By this we mean the *loss* of previous activities by a helper T-cell clone which can no longer enhance immunity (passive tolerance) and the *acquisition* of new abilities by which helper clones would no longer enhance immunity but rather actively inhibit it (active tolerance). Thus, we have shown that $CD4^+$ helper T cell clones lost the ability to undergo autocrine clonal expansion in phase II concomitant with loss of ability to secrete helper factors such as IL 2. In addition, we have since shown that such clones were also no longer able to produce helper factors enhancing B cell antibody secretion (Pawelec et al., Transplantation Proceedings, in press). On the other hand, they acquired the ability to induce suppressive cells via active stimulatory interactions governed by determinants that we have called "DY" (Pawelec et al., *J. Exp. Med. 167*:243, 1988). Hence, we suggest that both the lost and acquired functions could contribute to the establishment and maintenance of a tolerant state.