GENERATION OF DIVERSITY IN T CELL RECEPTOR REPERTOIRE SPECIFIC FOR PIGEON CYTOCHROME *c*

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T lymphocytes recognize foreign antigen in association with cell surface molecules encoded within the major histocompatibility complex (MHC). The T cell receptor (TCR)[1] complex that mediates this recognition is known to be composed of several polypeptide chains, two of which, the α and β chains, appear to participate directly in antigen and MHC molecule recognition (1–9). The genes that encode these two chains have recently been cloned and sequenced (10–14). Gene elements encoding both chains are organized in a fashion similar to immunoglobulin genes; variable (V), diversity (D), and joining (J) region gene segments rearrange to form a complete, functional gene. The presence of D regions in the α chain has not been determined, but the weight of evidence indicates that they are not prevalent, if they exist at all (12, 13, 15, 16).

Recent cell fusion and gene transfection experiments indicate that the T cell specificities for both antigen and MHC molecules reside in the α and β chains (17, 18). However, the molecular basis of MHC-restricted recognition remains to be elucidated, as does the influence of MHC on the T cell receptor repertoire. As an approach to understanding the structural basis of MHC-restricted T cell recognition of antigen, we have undertaken an extensive analysis of a T cell response defined in terms of both the fine specificity of antigen recognition and the fine specificity of MHC recognition.

The T cell response to pigeon cytochrome *c* has been informative in terms of defining the fine specificity of T cells for antigen and MHC molecules. Cytochrome *c* is a heme-containing mitochondrial protein 103–104 amino acids in length, and cytochrome *c* from pigeon differs from the murine protein at seven amino acid residues. Restimulation of pigeon cytochrome *c*–specific murine T cells by species variants of cytochrome *c* has indicated that the antigenic determinant lies at the COOH-terminal end of the molecule, and that amino acid substitutions at positions 99, 100, and 104 play an important role in functional recognition (19–23). This mapping of the antigenic determinant is also supported

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[1] *Abbreviation used in this paper:* TCR, T cell receptor.

	81							90								100			104
Mouse	Ile	Phe	Ala	Gly	Ile	Lys	Lys	Lys	Gly	Glu	Arg	Ala	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Lys	Ala	Thr	Asn	Glu
Pigeon								Ala											Gln			Ala	Lys	
Moth	Val				Leu		Ala	Asn											Gln			Lys	—	

FIGURE 1. COOH-terminal amino acid sequence of mouse cytochrome *c*, and the residues at which the pigeon and tobacco hornworm moth cytochromes *c* differ from the murine peptide.

by the fact that the COOH-terminal cyanogen bromide cleavage fragments 81–103 show patterns of both immunogenicity and antigenicity similar to that of the whole molecules (Fig. 1) (19, 20, 22). Synthetic peptide analogues of this COOH-terminal region have allowed for a more detailed analysis of the structural requirements for antigenic potency (23).

Immune responsiveness to pigeon cytochrome *c* has been mapped to the I-E subregion of the murine H-2 gene complex, and in particular to the more polymorphic I-E_β chain. Thus, MHC congenic strains that express $E^{k,s}_\beta$ are high responders to pigeon and moth cytochromes *c*, strains that express E^b_β are high responders to moth but not pigeon cytochrome *c*, and strains expressing other E_β alleles (e.g., $E^{d,p}_\beta$) are low- or nonresponders to either pigeon or moth cytochromes *c* (19, 22). Accordingly, T cells from three MHC-congenic strains, the B10.A (H-2^a), the B10.S(9R) (H-2^{t4}), and the B10.A(5R) (H-2^{i5}), respond to cytochromes *c* exclusively in association with I-E encoded Ia molecules.

Previous work has shown that the T cell proliferative response to pigeon cytochrome *c* in the B10.A strain of mice consists of a set of closely related clonotypes, all of which share specificity for a COOH-terminal antigenic determinant in association with the self E^k_α:E^k_β Ia molecule (22, 24, 25). The clonotypes can be distinguished based upon: (*a*) differences in the pattern of clonal activation by species variant cytochromes *c* (antigen specificity), (*b*) crossreactivities for cytochrome *c* recognition in association with allogeneic I-E-encoded Ia molecules (MHC restriction specificity), and (*c*) the reactivity of some cytochrome *c*–specific T cell clones for allogeneic MHC molecules in the absence of added antigen. Therefore, using different cytochrome *c* species-variant fragments and antigen-presenting cells (APCs) from different MHC-congenic strains of mice, the fine specificity of T cell clones can be defined in terms of antigen and MHC molecule crossreactions.

In this report, we have addressed questions concerning the structural basis of antigen-specific, MHC-restricted recognition. A series of T cell clones, categorized on the basis of antigen and MHC–specificity, were analyzed for the expression of α and β chain genes. Results of these experiments revealed that sequence changes in particular regions of the receptor can correlate with either MHC- or MHC and antigen–recognition, or both. As an adjunct to this analysis, we carried out prospective experiments in which heterogeneous T cell lines were selected on the basis of each of the different specificities, and the resulting T cell lines were analyzed for α and β chain gene expression. These experiments showed that T cell lines could be selected by growth in response to particular antigen/MHC combinations, and that this selection produced a limited diversity of T cells expressing the predicted α and β chain genes.

Materials and Methods

Animals. B10.A and B10.A(5R) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B10.S(9R) mice were bred at the National Institutes of Health (NIH), Bethesda, MD. Mice of either sex from 6 to 12 wk of age were used for experiments.

T Cell Lines and Clones. T cell lines were established from the draining lymph nodes of mice immunized 1 wk previously in the hind footpads with 100 μg pigeon cytochrome *c* emulsified 1:1 in complete Freund's adjuvant containing killed *Mycobacterium tuberculosis*, strain H37 Ra (Difco Laboratories, Detroit, MI). Long-term T cell lines were propagated as described previously (22) by repeated cycles of stimulation and rest in 24-well tissue culture plates (Nunclon; Thomas Scientific Co., Philadelphia, PA). T cell clones were derived by limiting dilution as described (22). The T cell hybridomas 2B4 and 2C2 were derived from the same line, while the 3.G8 clone was derived independently. Clones E2, A9, and A6 were derived from the same line, and clones C.F6, D2, C12, C8, and C10 were all derived from a separate line. Clones D4 and B10 were cloned from the same line. Clones 4.C3, 5C.C6, and 5C.C7 were derived from line 5.A.1 (see text). Clone 6.A.E1 was derived from line 6.A.1 (see text).

T cell proliferation assays were performed by culturing $1–2 \times 10^4$ T cells in flat-bottom 96-well microtiter plates with 5×10^5 irradiated (3,300 rad) spleen cells (APCs) and various concentrations of cytochrome *c* and cytochrome *c* COOH-terminal fragments 81–104 (prepared as previously described [26]). Proliferation was recorded as cpm of [^{3}H]-thymidine incorporated in a 12–18-h pulse with 1 μCi/well after 60 h of culture. Values represent the arithmetic mean (±SEM) of duplicate or triplicate cultures.

B10.A-derived pigeon cytochrome *c*–specific T cell lines were propagated by repeated in vitro stimulation with syngeneic APCs and antigen. However, T cell lines were also selected for particular specificities by stimulation with allogeneic APCs ± antigen as detailed in the text.

For generation of large numbers of cells, T cells were expanded for 3 d as previously described (27), in the presence of recombinant human interleukin 2 (IL-2) from *Escherichia coli* (28) (generously provided by Cetus Corp., Emeryville, CA) 48 h after antigen stimulation. The cells were then harvested, pelleted, and snap frozen in liquid nitrogen, and were used for preparation of high molecular weight DNA.

Preparation of High Molecular Weight DNA. $3–10 \times 10^7$ cells were resuspended in 25 ml of 10 mM NaCl, 10 mM Tris, 25 mM EDTA. Proteinase K and SDS were added to a final concentration of 50 μg/ml and 1%, respectively. The solution was incubated at 37°C for 3 h. After adding NaCl to a final concentration of 200 mM, the solution was extracted twice with phenol/chloroform (1:1), where the chloroform contained isoamyl alcohol at 24:1. This was followed by two ether extractions, and the DNA was precipitated with two volumes of cold ethanol and spooled onto a glass rod. The DNA was resuspended in 0.1× SSC (standard saline citrate) overnight at 4°C. Next, 50 μg/ml of heat-treated RNase A were added and the solution was incubated at 37°C for 30 min. Again NaCl was added to a final concentration of 200 mM and extractions were performed as above. After ethanol precipitation, the DNA was resuspended in 10 mM Tris, 0.1 mM EDTA.

Southern Hybridization. DNA from T cell lines and clones was digested to completion by manufacturers' specifications and electrophoresed on 0.8% agarose gels at 25 V for 36 h. The DNA was transferred to nitrocellulose by the method of Southern (29). Hybridization of the DNA probe was carried out at 42°C for 16 h in 5× SSPE (standard saline phosphate EDTA), 0.5% SDS, 1× Denhardt's (30), 50% formamide, 10% dextran sulfate, 100 μg/ml sheared and denatured salmon testes DNA. ^{32}P-nick-translated (31) DNA probe with a specific activity of $4–10 \times 10^8$ cpm/μg was added to a concentration of 10^6 cpm/ml. After hybridization, blots were washed once at room temperature and twice at 55°C for 20 min in 2× SSC, 0.1% SDS with a final wash in 0.2× SSC, 0.5% SDS. Labeled DNA fragments were visualized by autoradiography and sizes were calculated from a Hind III digest of phage λ DNA. After autoradiography, the probe was removed from the blot with two 15-min washes in 0.5 M NaCl, 50 mM NaOH, 2 mM EDTA at room temperature, and rehybridized as before with a different probe.

DNA Probes. The $D_\beta 1$, $J_\beta 1$, and $J_\beta 2$ region probes were isolated from three genomic clones kindly provided by Drs. N. R. J. Gascoigne and M. M. Davis, Stanford University, Stanford, CA (32, 33). The $D_\beta 1$ probe covers a 1.2 kb Pst I–Pst I fragment just 5′ of $J_\beta 1.1$. The $J_\beta 1$ probe includes $J_\beta 1.4$–$J_\beta 1.7\Psi$ on a 1.3 kb Sph I–Sac I fragment. The $J_\beta 2$ probe spans the whole $J_\beta 2$ cluster on a 1.2 kb Eco RI–Cla I fragment. The $D_\beta 2$ probe is a 0.82 kb Pvu II–Eco RI fragment 5′ of $J_\beta 2.1$, which was subcloned from a genomic clone kindly provided by Dr. J. Kaye, University of California, San Diego. These J probes will detect all β chain rearrangements, and the D probes will detect all D-J rearrangements that use the two known D_β regions (34, 35), when DNA is digested with Hind III or Eco RI.

The $V_\beta 2B4$ probe was subcloned from a cDNA clone kindly provided by Y.-h. Chien (33) by an Eco RI–Rsa I digest that yielded a 250 bp fragment. The remaining three V region probes were isolated from cDNA clones previously described (16). The $V_\beta B10$ probe was subcloned as a 450 bp Bam HI–Ava II fragment from the β chain cDNA of clone 4.C3. The $V_\beta C8$ probe was isolated as a 380 bp fragment by an Eco RI–Xmn I digest from the β chain cDNA clone of the T cell clone C8 (Fink, P. J., M.-L. Hsu, and S. M. Hedrick, unpublished results). The $V_\alpha 2B4.2$ probe was subcloned as a 400 bp Bam HI–Pst I fragment from the 4.C3 α chain cDNA clone. Arden et al. (36) have renamed $V_\alpha 2B4$ as $V_\alpha 11$, and Barth et al. (37) have renamed $V_\beta 2B4$ as $V_\beta 3$. The other variable region genes described in this report had not been identified at the time these variable region lists were published.

Results

Clonal Phenotypes Expressed in B10.A Mice

Antigen-specific, MHC-restricted T cell lines were established from B10.A mice immunized in vivo with pigeon cytochrome *c*. The lines were propagated by stimulation in vitro with antigen and syngeneic APCs and cloned by limiting dilution. Initially the specificity of each clone was examined by comparing the proliferation in response to pigeon cytochrome *c* in association with B10.A and B10.S(9R) APCs (Table I). A more detailed analysis comparing the proliferation in response to the COOH-terminal 81–103,4 fragments from pigeon and tobacco hornworm moth in association with three MHC-congenic splenic APC populations: syngeneic B10.A (E^k_α:E^k_β), B10.S(9R) (E^k_α:E^s_β), and B10.A(5R) (E^k_α:E^b_β) was also performed (Fig. 2). A comparison of the COOH-terminal amino acid sequences of mouse, pigeon and moth cytochromes *c* is shown in Fig. 1. Differences in responsiveness under these conditions allowed for the categorization of T cell clones into four related but distinct clonal phenotypes (Fig. 2, and Tables I and II).

Phenotype I. Clones of phenotype I, represented by E2, respond to pigeon cytochrome *c* and moth and pigeon fragments 81–103,4 in association with B10.A APCs, but not B10.S(9R) APCs. For many phenotype I clones, the dose/response curves for moth fragment are shifted >10-fold, to lower concentrations of antigen, as compared with pigeon fragment (16, 19, 20). However, for the clone E2 presented, there was only a modest shift detectable (Fig. 2). Phenotype I clones do not respond to pigeon or moth cytochromes *c* in association with B10.S(9R) APCs even at 24 μM (data not shown). In addition, phenotype I clones respond to moth fragment 81–103 in association with B10.A(5R) APCs (see clone E2 in Fig. 2).

Phenotype II. Clones of phenotype II represented by 5C.C6 share the anti-

TABLE I

Proliferation of B10.A Pigeon Cytochrome c–specific T Cell Clones

Phenotype	Clone	Strain of origin	[^{3}H]Thymidine incorporation* in response to pigeon cytochrome *c* (μM) on B10.A APCs:					
			24.0	2.4	0.24	0.024	0.0024	0
			cpm × 10^{-3}					
I	E2	B10.A	21.1 ± 1.8	192.5 ± 22.3	14.3 ± 1.3	2.9 ± 0.03	2.1 ± 0.1	2.1 ± 0.01
II	5C.C6	B10.A	1.6 ± 0.3	7.1 ± 1.2	15.8 ± 0.03	5.0 ± 0.3	0.2 ± 0.03	0.3 ± 0.05
IIIA	C12	B10.A	ND	7.7 ± 2.7	32.7 ± 0.2	11.8 ± 0.4	0.3 ± 0.1	0.4 ± 0.1
IIIB	D2	B10.A	ND	119.4 ± 18.7	234.2 ± 27.2	129.9 ± 1.4	0.9 ± 0.4	0.4 ± 0.2
IV	C10	B10.A	3.6 ± 0.5	6.2 ± 0.3	13.0 ± 1.4	31.2 ± 9.4	7.4 ± 0.2	0.3 ± 0.02
			[^{3}H]Thymidine incorporation* in response to pigeon cytochrome *c* (μM) on B10.S(9R) APCs:					
			24.0	2.4	0.24	0.024	0.0024	0
			cpm × 10^{-3}					
I	E2	B10.A	0.5 ± 0.04	0.7 ± 0.1	0.6 ± 0.05	0.6 ± 0.03	0.5 ± 0.0	0.7 ± 0.04
II	5C.C6	B10.A	13.2 ± 0.02	11.7 ± 3.2	18.5 ± 0.7	12.2 ± 0.3	16.2 ± 1.5	17.3 ± 0.3
IIIA	C12	B10.A	ND	2.5 ± 0.05	5.6 ± 3.2	8.7 ± 0.7	0.4 ± 0.02	0.8 ± 0.2
IIIB	D2	B10.A	ND	238.2 ± 17.7	238.1 ± 26.4	3.7 ± 0.9	0.5 ± 0.1	0.3 ± 0.2
IV	C10	B10.A	3.6 ± 1.3	1.6 ± 1.7	0.7 ± 0.3	0.6 ± 0.1	0.2 ± 0.02	0.2 ± 0.02

* T cell proliferation in response to the whole molecule of pigeon cytochrome *c*, assayed as described in Materials and Methods.

gen/MHC specificity of phenotype I clones in the presence of B10.A and B10.A(5R) APCs, but in addition proliferate in response to B10.S(9R) APCs in the absence of added antigen (Table I and Fig. 2). Clones of this type are thus alloreactive to B10.S(9R) APCs. Since these clones also proliferate in response to B10.S APCs, which lack I-E expression, we presume that the determinant recognized is $A^s_\alpha A^s_\beta$. In addition, one other phenotype II clone did not proliferate to A.TL spleen cells that express MHC molecules encoded by K^s and I^k, again suggesting the alloreactivity is specific for I-A^s (22). We have previously published the sequence and response of a similar clone 5C.C7 (16), and the evidence indicates that 5C.C6 and 5C.C7 are actually the same T cell clone. They were derived from the same cloning experiment, show the same alloreactive specificity, and have the same productive and nonproductive β chain gene rearrangements.

Phenotype III. Clones of phenotype III, represented by C12 and D2, respond to pigeon cytochrome *c* on both syngeneic B10.A and allogeneic B10.S(9R) APCs, and are not alloreactive in response to B10.S(9R) APCs (Table I). All clones of this type respond to pigeon and moth fragments in association with B10.A APCs, and to pigeon fragment in association with B10.S(9R) APCs. Clones of phenotype III can be further divided into two groups, IIIA and IIIB, based upon an antigen fine-specificity difference. Namely, phenotype IIIA clones (i.e., C12) respond well to the pigeon fragment but not the moth fragment with B10.S(9R) APCs, whereas phenotype IIIB clones (i.e., D2) respond to the moth fragment at least as well as the pigeon fragment when presented by B10.S(9R) APCs (Table I and Fig. 2). We have previously shown that two clones, B10 and 4.C3 (see Table II), representing the two variants of phenotype III, differ in receptor sequences at only the junction of the α and the β chain gene rearrangements (16).

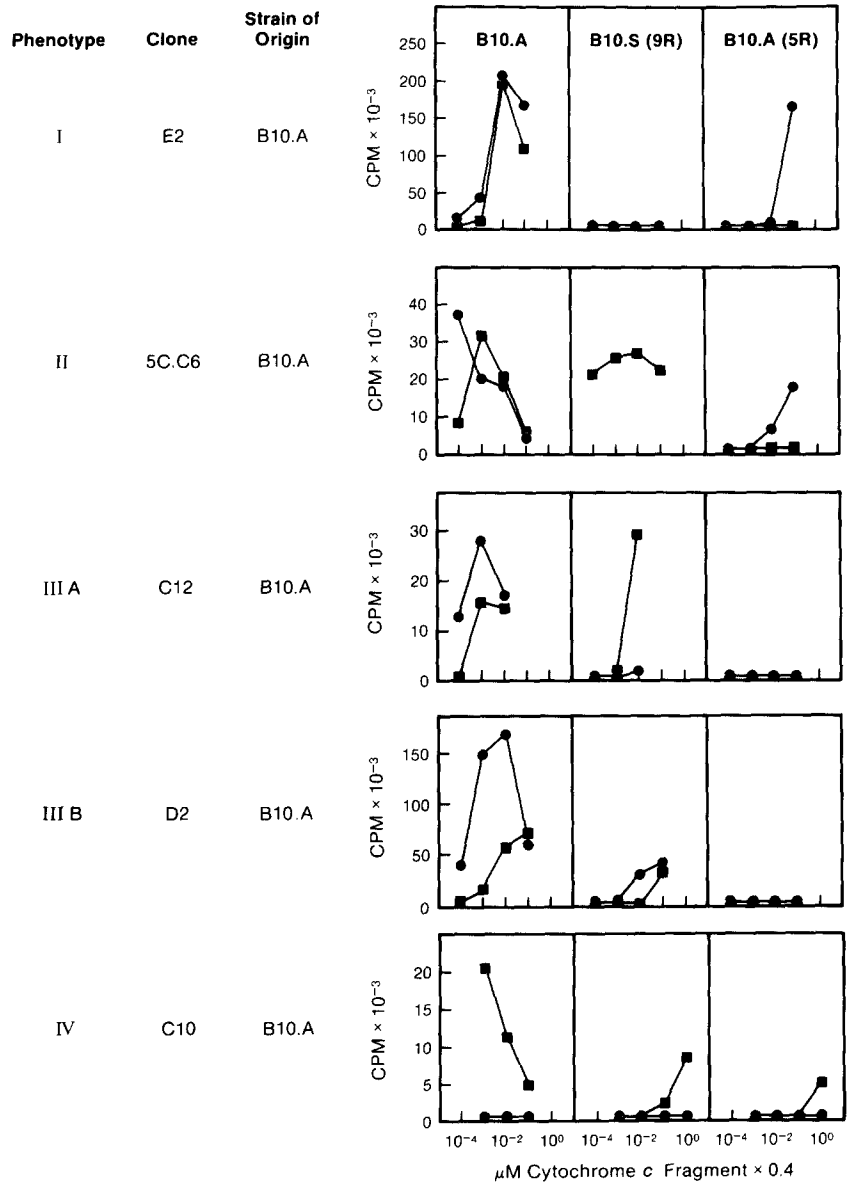

FIGURE 2. Proliferative responses of five prototype T cell clones, each exhibiting a distinct phenotype. Proliferation was measured as cpm of [^{3}H]thymidine incorporated as described in Materials and Methods. Antigens shown are pigeon fragment 81–104 (squares) and moth fragment 81–103 (circles). APCs were derived from either B10.A, B10.S(9R) or B10.A(5R) mice as indicated in the top panel. The response of clone 5C.C6 to B10.S(9R) APCs was independent of antigen.

When clones of phenotype III are compared with clones of phenotype I, the major difference in specificity is in the MHC crossreactivity. Phenotype I clones respond to cytochromes *c* in association with B10.A and B10.A(5R) APCs,

TABLE II

Clonal Phenotypes of B10.A Pigeon Cytochrome c–specific T Cells and their TCR Gene Rearrangements

Cell type	Name	V_β2B4 (Hind III)	V_βB10 (Eco RI)	V_βC8 (Hind III)	V_α2B4.2 (Hind III)	Phenotype	Figure
			kb				
B10.A clone	D4	4.3	—*	—	2.4	I	3 *A*
B10.A clone	C.F6	9.2	—	—	2.4	I	3 *A*
B10.A clone	6.A.E1	9.2	—	—	8.4	I	3 *B*
B10.A clone	E2	4.3	—	—	3.7	I	3 *B*
B10.A hybridoma	2C2	4.3	—	ND	3.6	I	3 *B*
B10.A hybridoma	2B4	4.3	—	—	2.6	I	3 *B*
B10.A clone	5C.C6	9.2	—	—	3.5	II	3 *A*
B10.A clone	5C.C7	9.2	—	—	3.5	II	6
B10.A clone	3.G8	—	6.0	—	2.4	IIIA	3 *A*
B10.A clone	C12	—	6.0	—	2.4	IIIA	3 *A*
B10.A clone	B10	—	6.0	—	2.4	IIIA	3 *A* and *B*
B10.A clone	D2	—	6.0	—	2.4	IIIB	3 *A*
B10.A clone	4.C3	—	6.0	—	2.4	IIIB	3 *A*
B10.A clone	C8	—	—	3.1	8.4	IV	3 *A*
B10.A clone	C10	—	—	8.5	8.4	IV	3 *A*
B10.A clone	A9	—	—	3.1	8.4	IV	3 *B*
B10.A clone	A6	—	—	3.1	8.4	IV	3 *B*

* No rearrangement was seen.

whereas phenotype III clones respond to cytochromes *c* in association with B10.A and B10.S(9R) APCs. The specificity for pigeon and moth cytochromes *c* in association with B10.A APCs appears to be nominally equivalent for the two phenotypes.

Phenotype IV. Clones of phenotype IV, represented by C10 in Table I and Fig. 2, proliferate in response to pigeon cytochrome *c* and pigeon fragment in association with B10.A (APCs), but do not respond to moth cytochrome *c* in association with any of the three APCs tested. In addition, phenotype IV clones respond to pigeon fragment at the highest concentrations of antigen (2.4 μM) in association both with B10.S(9R) and B10.A(5R) APCs. Thus phenotype IV clones differ from phenotype I clones both in antigen specificity, and in MHC specificity.

Correlations between α and β Chain Expression and Clonal Phenotypes

We have previously published the sequences for the productive rearrangements of T cell clones representing phenotypes I, II, IIIA, and IIIB (clones C.F6, 5C.C7, B10, and 4.C3, see Table II). Recently, we have also isolated and sequenced the productively rearranged α and β chain genes for a clone representing phenotype IV (clone C8). From our previous analysis, specificity differences were preliminarily ascribed to sequence changes in particular regions of the receptor (16). To determine whether these structure-function correlations were consistent for a larger set of T cell clones, a survey was undertaken of 14 additional clones representing phenotypes I, II, III, and IV. In this case, the α and β chain V-J combination of each clone was ascertained by the size of the

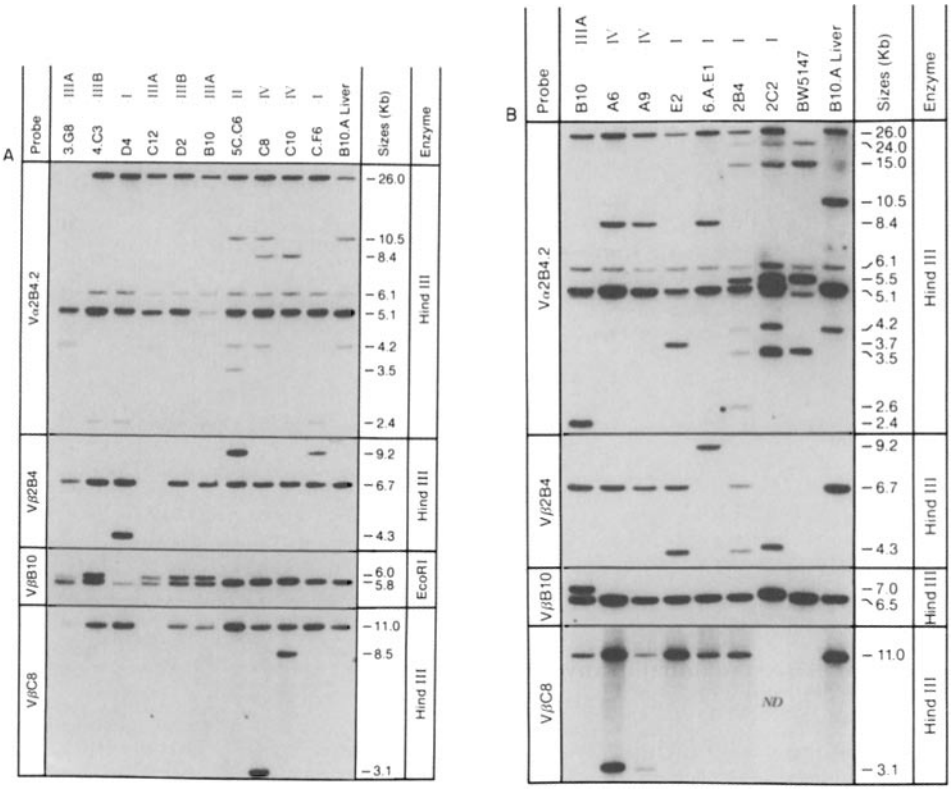

FIGURE 3. Southern blot analyses of T cell receptor α and β chain rearrangements. DNA from B10.A pigeon cytochrome *c*–specific T cell clones and hybridomas was digested with either Eco RI or Hind III as indicated. The response phenotype of each clone is indicated at the top of the lane. All lanes in each panel came from the same blot, hybridized with the indicated probe, with only irrelevant lanes removed. The probes are discussed in detail in Materials and Methods. (*A*) B10.A liver DNA was included as a control. The same blot was used for the V_α2B4.2, V_β2B4 and V_βC8 hybridizations. A separate blot was probed with V_βB10. (*B*) DNAs from B10.A liver and the hybridoma fusion partner, BW5147, were included as controls. V_α2B4.2, V_β2B4, and V_βB10 were used to probe the same blot. A separate blot was probed with V_βC8.

restriction fragment that hybridized to probes made from V region subclones. The V region probes hybridized to the productively rearranged band in the representative clones from which α and β chain genes were isolated. Each probe was designated by the T cell clone from which the V region was first isolated. Specific gene rearrangements were determined by Southern blot analysis (Fig. 3, *A* and *B*) and a summary of these rearrangements, grouped by clonal phenotype, is provided in Table II.

The α chain expressed in all the clones examined consisted of a member of a single family of crosshybridizing variable regions. We previously denoted this family by the T cell clone from which it was first isolated, 2B4, analogous to the way that immunoglobulin variable regions have been named. When further members of the family were isolated, we have distinguished them by numerical

designations (16). Thus, V_α2B4.1 was the first member of the variable region gene family to be isolated, and V_α2B4.2 was the second. Among the six phenotype I clones, five different rearrangements that hybridized to the V_α2B4.2 probe were seen (Fig. 3, *A* and *B*; summarized in Table II). The genes expressed by rearrangements resulting in 2.4, 2.6, and 8.4 kb Hind III bands have been sequenced, and represent V_α2B4.2-J_α84 (16), V_α2B4.1-J_α2B4 (38) and V_α2B4.2-J_αTA28 (Fink, P. J., M.-L. Hsu, L. A. Matis, and S. M. Hedrick, unpublished sequence), respectively (J_αTA28 is from Arden et al. [36]). The other two distinct rearrangements at 3.6 and 3.7 kb have not been analyzed at the sequence level, and therefore represent unknown V_α2B4-J_α combinations. We have not discerned an effect on the cytochrome *c*/MHC specificity of phenotype I T cell clones that relates to these different V_α2B4-J_α combinations.

As seen from the analysis of the Southern hybridizations in Fig. 3 and summarized in Table II, the expression of V_β2B4 appears to be important for phenotype I specificity. V_β2B4 is a single gene family in the B10.A strain (33), and the two hybridizing Hind III restriction fragments represent the same variable region gene rearranging to J_β1.2 (9.2 kb) and J_β2.5 (4.3 kb) (16, 33). All six phenotype I clones express one of these two V_β2B4-J_β combinations. Overall, this analysis indicates the importance of the V_α, V_β, and J_β regions in determining the antigen/MHC specificity characteristic of phenotype I. Of course, from this type of analysis junctional or diversity region sequence differences that may also affect the T cell specificity cannot be discerned. We have demonstrated one example of the effects of junctional sequence differences on the fine specificity of pigeon cytochrome *c*–specific clones elsewhere (16).

Two T cell clones characteristic of phenotype II (5C.C6 and 5C.C7) have been analyzed in this report, and as stated above, there is good reason to believe that these two clones came from the same progenitor cell. This phenotype is common among cytochrome *c*–specific B10.A clones, and as shown in detail in the next section, clones with I-A^s alloreactivity can be readily selected in culture. From our original analysis (16), we predicted the difference in receptor sequence that appears to distinguish phenotypes I and II is in the J_α region. Of the J_α genes that may be used in the pigeon cytochrome *c* response, we suggested that the selection of J_αC7 (16) appeared to change the overall conformation of the receptor such that an I-A^s alloreactivity was revealed. As shown in Fig. 3*A*, the Hind III band representing rearrangement of V_α2B4.2 to J_αC7 is 3.5 kb (clones 5C.C6 and 5C.C7).

All the clones of phenotype III manifested identical V_α and V_β rearrangements (Fig. 3*A* and Table II). Sequence data from clones B10 and 4.C3 showed that these clones are using V_α2B4.2 and J_α84 (16), which results in the 2.4 kb Hind III band shown in Fig. 3*A*. As noted, this rearrangement is also seen in some clones of phenotype I (e.g., C.F6). Similarly, all the clones characteristic of phenotype III rearranged their DNA to give a 6.0 kb Eco RI fragment when probed with V_βB10. This band corresponds to V_βB10 rearranged to J_β2.1 (16). Thus, the most striking difference between phenotype I clones and phenotype III clones is in their V_β-J_β gene expression. T cell clones of phenotypes I and III express distinctive and restricted sets of V_β-J_β genes, and the association of these

alternate β chains with a V_α2B4 gene family α chain accounts for the marked difference in MHC specificity.

The four clones of phenotype IV (C8, C10, A6, and A9) rearrange a third noncrosshybridizing V_β in association with a V_α2B4 family gene. The β chain for the clone C8 has been isolated from a cDNA library and sequenced. This clone is using a previously unidentified V_β, called here V_βC8, and J_β2.4 (Fink et al., unpublished sequence). Clones C8, A9, and A6 are all using the same β chain rearrangement, which gives a 3.1 kb Hind III band hybridizing to the V_βC8 probe on a Southern blot (Fig. 3, *A* and *B*). Clone C10 also uses V_βC8, but from the size of the hybridizing band, namely 8.5 kb (Fig. 3*A*), it must be using a J_β region from the first J cluster. All four clones rearrange V_α2B4.2, displaying the same 8.4 kb Hind III rearrangement as phenotype I clone 6.A.E1. Because 6.A.E1 responds to moth cytochrome *c* with B10.A APCs (data not shown), the failure of all four phenotype IV clones to respond to the moth fragment can most likely be attributed to the association of V_βC8 with the V_α2B4.2 gene. Thus, alternate V_βs associating with similar V_αs may affect either MHC (phenotype I vs. III), or antigen plus MHC (phenotype I vs. IV) specificity. The possibility exists that the unique MHC or antigen specificities described for phenotype IV clones come from junctional differences in either of the α or β chains, but the consistent correlation of V_βC8 expression with the overall phenotype (four out of four) makes that possibility unlikely.

Growth Selection of B10.A T Cell Lines for Distinct Phenotypes

Cytochrome *c*–specific proliferative T cells may be propagated in vitro by stimulation with antigen and APCs in the absence of exogenous growth factors, followed by further expansion in the presence of IL-2. By selecting the antigen and APCs used to grow the T cells, uncloned populations can be enriched for clones expressing particular reactivities. The gene rearrangements present in such mixed T cell populations can then be analyzed. This procedure can be used to determine the extent of detectable heterogeneity within the population of pigeon cytochrome *c*–specific T cells, and to determine whether selection at the population level for particular specificities correspondingly selects for T cells expressing predicted V_α and V_β rearranged genes. As stated above, the majority of B10.A pigeon cytochrome *c*–reactive cells exhibit phenotype I. Therefore, the α and β chain genes used by clones of this phenotype should be predominantly rearranged in the DNA of pigeon cytochrome *c*–specific T cell lines. From the T cell clones we have previously isolated and studied, we know that these lines also contain T cells characteristic of phenotypes II, III, and IV, thus, the genes expressed by these clones should be rearranged as well. B10.A pigeon cytochrome *c*–specific T cell lines were initially propagated by stimulation with syngeneic APCs and antigen. Subsequent stimulations were performed to select for reactivities characteristic of the distinct clonal phenotypes.

To detect all rearrangements at the β chain locus, we used single-copy genomic probes isolated from the two J_β clusters, J_β1 and J_β2 (see Materials and Methods). Among these rearrangements will be DJ rearrangements, incapable of producing a productive transcript, and VDJ rearrangements, including those that are both

productive and unproductive. To eliminate the obviously unproductive DJ bands from our analysis, two probes were made from DNA upstream of each of the two known D regions of the β chain gene (see Materials and Methods; see also references 34 and 35). These upstream DNA regions would be deleted if a V region was joined to the D region by a deletional mechanism. Therefore, when a rearranged band hybridizes with one of these D probes, the band cannot be the product of a complete VDJ rearrangement. If rearrangement has occurred by an inversion mechanism, or by sister chromatid exchange (39), the D probe could still hybridize to a DNA fragment, but not the same size fragment as that which hybridizes to the J probe.

Similar analysis of the α chain locus is not as easily carried out due to the fact that J_α gene elements are spread over at least 60 kb of DNA (15, 40), a size difficult to analyze by standard agarose gel electrophoresis. Therefore, our analysis of the α chain is limited to probes for the V_α regions. However, since all the cytochrome *c*–specific B10.A clones examined thus far use members of the same crosshybridizing V_α gene family, our analysis of the α chain, though restricted to the V_α2B4.2 probe, reflects a majority of the cytochrome *c*–specific T cells in the population.

The analysis of β and α TCR gene rearrangements in cytochrome *c*–specific T cell lines selected for distinct functional specificities is shown in Figs. 4 and 5, respectively. Using the genomic probes that hybridize to the two J regions of the β chain, DNA from each line was analyzed. Rearrangements that represent less than ten percent of the total population can be detected (data not shown). The bands that were determined to be DJ rearrangements using the D probes are marked with a dot. The previous probes were stripped and the blots were rehybridized with one of three V_β probes, V_β2B4, V_βB10, and V_βC8, which we considered important for the pigeon cytochrome *c* response from our clone data. Bands that hybridized to one of the V_β region probes are numbered (Fig. 4).

Cytochrome c–specific T Cell Lines Have Predominantly the Rearrangements of Phenotype I. Line 5.A.1 was derived from B10.A mice immunized with pigeon cytochrome *c*. The line was stimulated six times in vitro with the pigeon cytochrome *c* on B10.A APCs. We would expect the majority of the cells in this line to exhibit phenotype I. As shown in Fig. 4, there is a prominent 3.7 kb Eco RI band in line 5.A.1 that hybridized to the J_β1 probe (labeled *1* in Fig. 4). This band also hybridized to V_β2B4 and corresponds to the 9.2 kb Hind III band seen in the T cell clones 6.A.E1, C.F6, 5C.C6, and 5C.C7. This band represents V_β2B4 rearranging to J_β1.2. A faint band at 1.7 kb, which hybridized to the V_β2B4 probe and J_β2, could be seen on a longer exposure. This rearrangement represents the V_β2B4-J_β2.5 rearrangement seen in the T cell hybridomas 2B4 and 2C2, and clones D4 and E2. The J_β2 and V_βB10 probes hybridized to a faint band at 6.0 kb (labeled *2* in Fig. 4). This line also has two other VDJ rearranged bands, one at 2.4 kb, which hybridized to the J_β2 probe, and the other at 11.0 kb, which hybridized to the J_β1 probe. Neither of these bands hybridized to the three V_β region probes we tested. These bands may represent unproductive VDJ rearrangements carried by a prominent clone in this line or rare productive VDJ

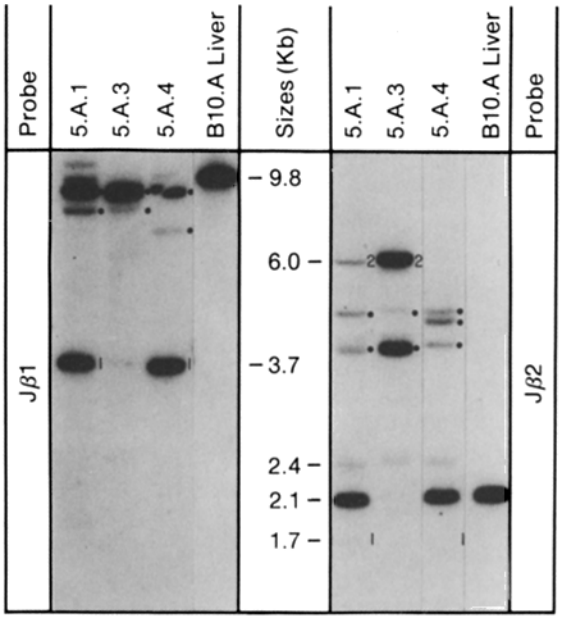

FIGURE 4. Southern blot analyses of three pigeon cytochrome *c*–reactive T cell lines selected for particular specificities. As described in the text, line 5.A.1 was stimulated with antigen on B10.A APCs, line 5.A.3 was stimulated with antigen on B10.S(9R) APCs, and line 5.A.4 was cultured with B10.S APCs in the absence of antigen. The DNA from these lines and B10.A liver was digested with Eco RI, and the two blots represent duplicate samples run on the same agarose gel. The blots were hybridized with probes to the two J_β clusters, $J_\beta 1$ and $J_\beta 2$, and then reprobed with V_β and D_β probes (see Materials and Methods). Those bands that also hybridized to one of the D probes, and therefore represent aberrant DJ rearrangements, are marked with a dot (●). Bands that subsequently hybridized to $V_\beta 2B4$ (1) and $V_\beta B10$ (2) are also marked.

rearrangements of clones that have not yet been described. None of the rearranged bands in this line hybridized to the $V_\beta C8$ probe.

When line 5.A.1 was analyzed for V_α rearrangements, the most prominent Hind III band seen was 2.4 kb (Fig. 5). This is the same rearrangement used by two of the phenotype I clones, D4 and C.F6, and all the phenotype III clones. Three other rearranged bands are apparent in this line. Two of these, the 3.7 and 2.6 kb bands, correspond to rearrangements seen in the phenotype I clones, E2 and 2B4 respectively (see Fig. 3*B* and Table II). The third band is 3.5 kb, which is the same band seen for the phenotype II clones, 5C.C6 and 5C.C7. That these rearrangements represent common patterns of cytochrome *c*–specific T cell lines is further supported by the fact that the T cell clones analyzed in Fig. 3 *A* and *B* were derived from seven independent T cell lines (including line 5.A.1), and most of these clones express α chain genes rearranged to give one of the Hind III fragments described above. The most prominent overall rearrangement pattern for 5.A.1 is most like clone C.F6, which is phenotype I. To a lesser extent, the alternate $V_\beta 2B4$-$J_\beta 2.5$ rearrangement of phenotype I, as well as the rearrangement patterns of phenotype II and III T cell clones, are also observed (see Table II). Since no rearranged bands were detected with the $V_\beta C8$

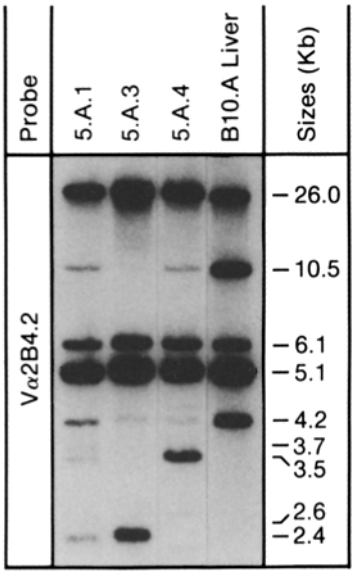

FIGURE 5. α chain gene rearrangements in selected T cell lines. DNA from the same T cell lines as in Fig. 4 was digested with Hind III and analyzed on a Southern blot with the V_α2B4.2 probe described in Materials and Methods.

probe and the 8.4 kb rearrangement of V_α2B4.2 was not seen, it appears that clones of phenotype IV are not prevalent in this line. The low frequency of cloning phenotype IV clones would also indicate that these clones are rare in the pigeon cytochrome *c* response.

T Cell Lines Selected for Phenotype III. Since the clones of phenotype III respond to antigen on B10.S(9R) APCs while phenotype I clones do not, stimulation of the line by the addition of pigeon cytochrome *c* and B10.S(9R) APCs should select for T cells that have a 6.0 kb V_βB10 rearrangement. For this analysis, line 5.A.3 was derived from line 5.A.1 at the second in vitro stimulation, and was subsequently stimulated with B10.S(9R) APCs and pigeon cytochrome *c*. After four generations of selection, this line manifested the proliferative specificity characteristic of clones of phenotype III (data not shown). Using the J_β1 and J_β2 probes to analyze the β chain locus in line 5.A.3 (Fig. 4), the prominent rearranged band was a 6.0 kb Eco RI fragment that hybridized to both J_β2 and V_βB10 (labeled *2* in Fig. 4). There are no detectable rearranged bands that hybridized to V_β2B4 or V_βC8. This rearrangement pattern is identical to that of all the clones with phenotype III (Fig. 3*A*). It should be noted that this line displays two prominent D_β-J_β rearrangements and no germline band, implying the prevalence of one clone. However, two additional minor D_β-J_β rearrangements, as well as a 2.4 kb VDJ rearrangement, which hybridizes to the J_β2 region, are also seen, indicating the presence of at least one other clone. At the α chain locus, line 5.A.3 seems to have only one rearranged V_α2B4 band of 2.4 kb (Fig. 5). Thus, a B10.A T cell line selected in vitro for reactivity to pigeon cytochrome *c* presented by allogeneic B10.S(9R) APCs displayed the rearrangements of both α and β chain TCR genes that were characteristic of all the phenotype III clones.

TABLE III

Proliferation of Pigeon Cytochrome c–specific T Cell Lines after In Vitro Selection for I-A^s-specific Alloreactivity

APCs	Antigen concentration	[^{3}H]Thymidine incorporation* in:		
		5.A.4	6.A.2.10	6.A.4.13
	μM		$cpm \times 10^{-3}$	
B10.A	2.4	7.2 ± 0.7	14.0 ± 0.6	56.6 ± 4.0
	0.24	23.4 ± 5.2	25.0 ± 0.6	92.9 ± 15.3
	0.024	42.2 ± 3.5	30.9 ± 8.3	4.3 ± 0.1
	0.0024	20.8 ± 5.3	18.4 ± 1.8	2.0 ± 0.4
	0	4.3 ± 0.7	1.0 ± 0.01	1.5 ± 0.8
B10.S	0	31.6 ± 7.1	0.9 ± 0.1	75.8 ± 6.1

* Assay performed as described in Materials and Methods.

Phenotype II clones, which are alloreactive to B10.S(9R) APCs, were not expanded in this T cell line. This may be due to a low precursor frequency of these clones in the initial population, or to a relatively low reactivity of phenotype II clones under these stimulation conditions.

T Cell Lines Selected for Phenotype II. A correlation between alloreactivity and the use of J_αC7 in phenotype II clones has been observed previously (16). If the expression of this J_α gene element accounted for the alloreactivity manifested by phenotype II clones, we would expect the 3.5 kb V_α2B4 band to become prevalent in line 5.A.1 after stimulation with allogeneic I-A^s-bearing APCs. For this purpose, line 5.A.4 was derived from line 5.A.1 at stimulation 5 by three subsequent stimulations with B10.S (H-2^s) APCs in the absence of cytochrome *c*. This line, the origin of the clones 5C.C6 and 5C.C7, displayed strong alloreactivity to the A^s_α:A^s_β molecule as well as specificity for cytochrome *c* in association with B10.A APCs (Table III). The pattern of rearrangements at the β chain locus for line 5.A.4 was very similar to line 5.A.1 (Fig. 4). A very obvious EcoRI band at 3.7 kb hybridized to both the V_β2B4 and the J_β1 probes. After longer exposure, a faint band at 1.7 kb was also detected with the V_β2B4 and J_β2 probes. In addition, the 2.4 kb band that hybridized to the J_β2 probe but not the three V_β probes was present in this line as well as the two previously described lines (5.A.1 and 5.A.3). In this line, there was no hybridization of any rearranged band to the V_βB10 or V_βC8 probes. Hybridization with V_α2B4.2 showed a prominent 3.5 kb Hind III band in line 5.A.4, as well as three other distinct bands which were not seen in any of the clones (Fig. 5). Since line 5.A.4 has this prominent 3.5 kb V_α2B4 band in addition to the prominent β chain rearrangement described, the majority of the cells exhibit the rearrangement pattern of the phenotype II clone 5C.C6 (Fig. 3*A* and Table II) following selection for I-A^s alloreactivity.

As clones 5C.C6 and 5C.C7 were derived from line 5.A.4, we had not independently confirmed that I-A^s alloreactivity was conferred by the V_α2B4.2-J_αC7 rearrangement. Perhaps numerous J_α substitutions would yield this phenotype, or junctional sequence changes in the β chain might be responsible. To determine if the presence of J_αC7 was necessary for the phenotype II alloreactiv-

ity, an independently derived T cell line, 6.A.2, was selected for B10.S reactivity. After four cycles of in vitro stimulation with antigen and syngeneic B10.A APCs, the subline 6.A.4 was derived by selection for alloreactivity to the A^s_α:A^s_β Ia molecule. Line 6.A.4 was analyzed after either four, seven, or nine cycles of stimulation with B10.S APCs without antigen (designated 6.A.4.8, 6.A.4.11, and 6.A.4.13, respectively). The proliferative responses of the previously described alloreactive line 5.A.4, the newly established alloreactive line 6.A.4.13, and line 6.A.2 after 10 stimulations in vitro with antigen and syngeneic B10.A APCs (6.A.2.10) are presented in Table III. The T cell line 6.A.2.10 manifested no detectable reactivity to B10.S APCs. However, after nine cycles of selection with B10.S APCs, the magnitude of proliferation of subline 6.A.4.13 to B10.S APCs was as much as 70% of the antigen-induced response. Line 5.A.4, as described above, also proliferated strongly with the addition of allogeneic B10.S APCs in the absence of antigen, and with the addition of syngeneic B10.A APCs plus optimal concentrations of cytochrome *c*. Thus, if the 3.5 kb V_α-J_α rearrangement seen in phenotype II clones was necessary for alloreactivity, we would expect this to be the prominent α chain rearrangement in line 6.A.4 as well.

The genomic DNA from lines 6.A.4.8, 6.A.4.11, and 6.A.4.13, as well as 5.A.4 and the control line 6.A.2.10, was analyzed on a Southern blot with the V_α2B4.2 probe. The predominant V_α2B4 Hind III rearrangement of the 6.A.2 line stimulated with antigen and syngeneic B10.A for 10 cycles (6.A.2.10) was the 8.4 kb band (Fig. 6). It is not clear why selection for phenotype I in this line resulted in this prominent rearrangement, while the identical selection of line 5.A.1 resulted in a predominance of the 2.4 kb V_α rearrangement, but as noted above, these alternate V_α-J_α rearrangements do not appear to have a noticeable effect on the specificity of phenotype I clones. Upon repeated stimulation of line 6.A.4 with B10.S APCs in the absence of cytochrome *c*, a 3.5 kb Hind III band became apparent and increased in intensity with repeated stimulations, while the 8.4 kb band became less prominent (Fig. 6). Thus, the same 3.5 kb Hind III band was observed as in the previously established I-A^s-reactive cytochrome *c*–specific T cell line 5.A.4 and the 5C.C7 clone derived from this line (lanes *1* and *2* in Fig. 6). This experiment was carried out a third time, and again selection by B10.S APCs resulted in the appearance of a 3.5 kb Hind III band hybridizing to V_α2B4.2 (data not shown). Thus, the progressively increased representation of the 3.5 kb V_α2B4 band accompanied by the concurrent acquisition of I-A^s alloreactivity independently reproduced the expression of V_α2B4-J_αC7, as in the previously derived phenotype II clones 5C.C6 and 5C.C7, and the 5.A.4 line.

Discussion

The T cell proliferative response to the well-defined protein antigen pigeon cytochrome *c* in the B10.A strain of mice yielded clones of a limited number of fine specificities. Since the clones exhibited unique crossreactivities in MHC and antigen recognition, we were able to define four related but distinct response phenotypes. The expression of a limited number of germline TCR elements in the overall response made possible the analysis of the molecular basis for each fine specificity by using DNA probes to particular components of the T cell

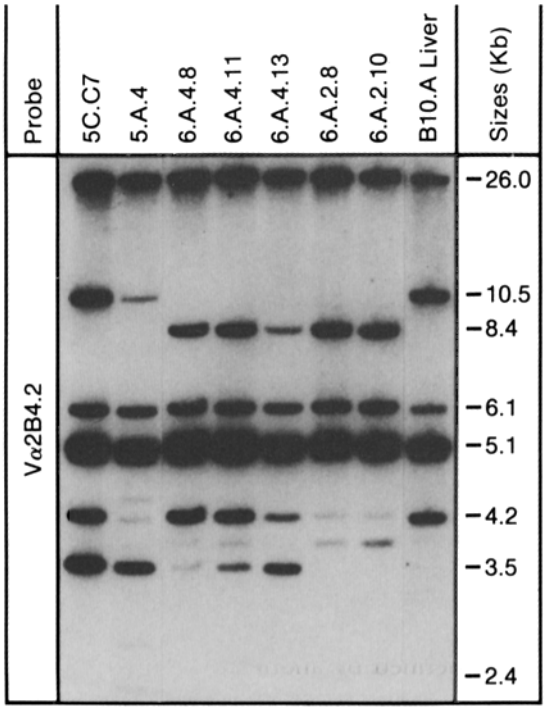

FIGURE 6. α chain rearrangements in T cell lines alloreactive for I-A^s. DNA from three pigeon cytochrome *c*–specific T cell lines selected for alloreactivity to I-A^s and two unselected lines was digested with Hind III and analyzed on a Southern blot. DNA from the phenotype II clone 5C.C7, line 5.A.4 and B10.A liver was added for comparison.

receptor α and β chains. This type of analysis also allowed us to make certain conclusions about the structural basis for the generation of diversity in the T cell receptor repertoire.

An analysis of gene rearrangements of T cell clones from B10.A mice, derived from seven independent immunizations, revealed the use of members of a single crosshybridizing V_α gene family expressed in association with three non-cross-hybridizing V_β genes. In combination with these variable region genes, only a limited number of J regions at both the α and β chain loci was used by these clones. Within an individual phenotype, at most 5 out of 50 J_α regions were used, and for each V_β gene, 1 or at most 2 different J_β genes out of a total of 12 were used. The diversity of J region expression within each phenotype varied. For example, all the clones of phenotype III used identical V_α-J_α and V_β-J_β rearrangements, while clones of phenotype I used two J_βs and five J_αs. The presence of five distinct V_α-J_α rearrangements among the six phenotype I clones analyzed in this report (Table II) does not necessarily imply that the J_α segment was not selected. For example, in the pigeon cytochrome *c*–specific T cell line 5.A.1, we found three of these five V_α-J_α rearrangements represented, although none of the phenotype I clones analyzed were derived from this line. A second pigeon cytochrome *c*–specific T cell line 6.A.2.8 (see Fig. 6) showed the 8.4, 3.7, and

2.4 kb rearrangements of V_α2B4.2. Recent molecular analysis of a third independently established cytochrome *c*–specific T cell line has revealed the reoccurrence of the same V_α-J_α rearrangements as seen in line 6.A.2.8 (Matis, L. A., and S. B. Sorger, unpublished data). Therefore, the V_α-J_α rearrangements used by phenotype I clones are clearly reproducible. Selection for J segments was also observed in the four phenotype IV clones, which were derived from two separate immunizations, and all of which displayed the same V_α-J_α rearrangement and one of two V_βC8-J_β rearrangements. Thus, all the V and J regions making up the T cell receptor were selected, implying the importance of each element in determining the overall specificity for antigen and MHC molecules. However, among the limited set of gene elements expressed by any one phenotype, we do not see any effects on fine specificity. For example, the specificity of clones within a phenotype expressing different J_α and J_β gene elements in association with the same V_α and V_β genes are not distinguishable based on antigen/MHC specificity by our criteria.

All the B10.A pigeon cytochrome *c*–specific T cell clones and lines studied use a member of the V_α2B4 gene family, indicating a critical role of these genes in the recognition of the COOH-terminal antigenic determinant in association with the E^k_α:E^k_β Ia molecule. There is overlap in the use of specific α chain rearrangements such that the V_α-J_α combinations used by clones of one phenotype may be used in the recognition defined by another phenotype. For phenotypes I, III, and IV, there was a distinct difference in the MHC specificity of the clones as determined by the crossreactive recognition of MHC molecules in association with antigen. These phenotypes correlated perfectly with the V_β-J_β combination expressed by the clones: phenotype I clones recognized antigen in association with E^k_α:E^k_β and E^k_α:E^b_β and expressed V_β2B4, phenotype III clones recognized antigen in association with E^k_α:E^k_β and E^k_α:E^s_β and expressed V_βB10, and phenotype IV clones recognized antigen in association with E^k_α:E^k_β, E^k_α:E^s_β and E^k_α:E^b_β and expressed V_βC8. However, phenotype IV differs from the other phenotypes in antigen as well as MHC recognition, as demonstrated by the nonresponsiveness of phenotype IV clones to moth cytochrome *c*. This result clearly shows that, for this response, the β chain can influence both antigen and MHC recognition. Thus, the predominant specificity differences among phenotypes I, III, and IV can be attributed to combinatorial association whereby alternate β chains are associated with the same α chain. Functional experiments have suggested that the difference in antigenicity between moth and pigeon cytochromes *c* is accounted for by the presence of the COOH-terminal lysine at residue 103 rather than 104 (19, 23, 41, 42). One model proposes that these substitutions appear to affect the interaction between antigen and the MHC molecule and not directly the interaction between antigen and the TCR (41–43). Thus, it is possible that phenotype IV clones have a unique MHC specificity that only indirectly affects the antigen specificity of the clones. Experiments are in progress to determine whether phenotype I and phenotype IV clones differ in the recognition of cytochromes *c* substituted at residue 99, the putative residue involved in the antigen-TCR interaction (19, 20, 42, 43).

In previous work (16), we made the argument that the alloreactivity of clone

5C.C7 (phenotype II) as compared to C.F6 (phenotype I) was due to the J_α difference. This was based on the observation that the fine specificity of 5C.C7 and C.F6 differed essentially only the presence of alloreactivity specific for I-A^s in the 5C.C7. The primary structure of the receptors of these two clones showed that their TCR α and β chains differed by only a single amino acid in the D_β region, and by the usage of two different J_α regions. Since the major difference was in the J_α gene element, we proposed that in this case, a change in J_α accounted for the addition of alloreactivity to the specificity of 5C.C7. Herein, we have presented experiments in which cells expressing this alloreactivity were selectively expanded from a heterogeneous antigen-specific population. In three separate experiments, cytochrome *c*–specific T cells from independently immunized mice were selected for alloreactivity to I-A^s and this selection expanded a population of cells with the characteristic V_α2B4.2-J_αC7 rearrangement. Repeated stimulation of the initial population with antigen and syngeneic APCs did not produce any detectable levels of this rearrangement. Therefore, we conclude that in these pigeon cytochrome *c*–specific clones, alloreactivity for I-A^s correlates with a combinatorial difference in the α chain, and this combinatorial difference is relatively frequent and necessarily endows the T cells, otherwise representative of phenotype I, with an I-A^s-specific alloreactivity. Since there are several J_α regions used by phenotype I clones which exhibit identical antigen fine specificities in our analysis, it may not be unreasonable to assume that these J_αs may also correlate with other specific alloreactivities if an extensive analysis was carried out. Such a result would be consistent with the concept that the antigen-specific and alloreactive repertoires extensively overlap (44, 45). Studies on cytochrome *c*–specific clones with newly defined alloreactivities are presently being conducted to analyze whether similar combinatorial mechanisms account for alloreactivity in antigen-specific T cells in general.

In our previous paper (16), two clones that exhibited phenotype IIIA and IIIB, respectively, were analyzed at the sequence level. These two prototype clones, B10 and 4.C3, differed by only three amino acids, all at the rearrangement junctions of their TCR genes. Therefore, these differences must have accounted for the variation in fine specificity. In this paper, we have analyzed five clones of phenotype III, including B10 and 4.C3, derived from four independent immunizations. The three new clones segregated on the basis of exactly the same fine specificity differences such that they could be grouped as either phenotype IIIA or IIIB. The analysis in this report has shown that all these phenotype III clones use identical V and J regions for both chains of the TCR. Since the selection of the same variants of phenotype III, using identical gene elements, is reproducible, we would predict that the difference in the fine specificity must again be attributed to junctional diversity. Paradoxically, N-region and junctional differences that are thought to be highly variable are giving rise to two discrete and reproducible phenotypes. These results may imply the selection of N regions in response to pigeon cytochrome *c* and B10.A APCs, since these are the stimulating ligands. The selection would allow two types of N-region sequences to be used and their individual usage would manifest the fine-specificity difference seen in response to antigen and B10.S(9R) APCs. Alternatively, selection may occur at

the DNA level, such that the addition of nucleotides at the N regions would not be random. There is precedent for selection of N regions at both these levels in immunoglobulins (46). We are in the process of sequencing these new clones to determine which N regions are being used and which may be essential for each variant.

The data presented in this paper can be interpreted by drawing an analogy to the diversity of the immunoglobulin genes expressed during antibody responses to specific antigens. For example, the antibody response to phosphorylcholine (PC) in BALB/c mice is perhaps the most limited in diversity, and consists almost entirely of a single V_H-J_H combination (V_HT15-J_H1) associated with at least three completely different V_κ genes, all of which rearrange to J_κ5 (47). In this response the V_H-J_H combination and the J_κ gene elements are selected; however the V_κ response appears to consist of different, relatively nonhomologous genes that do not appreciably affect the affinity of the antibody for phosphorylcholine. In response to the hemagglutinin protein of influenza virus (48), at least three different V_H genes are expressed in combination with three out of four J_H elements. The selected portion of the antibody molecule is clearly the V_κ21C gene that is expressed by all hybridoma antibodies isolated thus far. The J_κ gene elements joined to V_κ21C consists of three out of five possible J_κs. The expression of the different V_H genes does not appear to influence the binding specificity of the antibodies for the influenza hemagglutinin determinant. Similarly, the antibody responses to arsonate and oxazolone are both heterogeneous with respect to one or more of the gene elements making up the expressed antibody molecules (49). In contrast, the T cell response to pigeon cytochrome *c* is selected with respect to the expression of both V_α and V_β, and the J regions used for both chains are from a limited subset of the total. The V_α response consists of a single V_α gene family, and the differences in the V_β genes can be shown to dramatically affect the specificity of the resulting T cell clones. This T cell response may be fundamentally different from the antibody responses thus far examined, in that all of the gene elements involved in receptor diversity appear to be selected, and each may contribute to the specificity or affinity of the response.

Another potentially fundamental distinction between T cell and B cell receptor diversity is illustrated by further consideration of the antibody response to influenza hemagglutinin (48). As noted above, combinatorial mechanisms contributed to sequence diversity for both heavy and light chains, but appeared to have little influence on specificity. The predominant effects on the fine specificity of antigen binding were attributed to somatic mutations accumulated within the V_κ21C gene. In contrast, our sequence analysis of four cytochrome *c*–specific T cell clones (16), as well as evidence from previous reports (33, 50), suggests that somatic mutation does not play a significant role in the generation of TCR diversity. Since one consequence of selected somatic mutations is to increase the affinity of the antibody molecule for its ligand (49), it would not be surprising if a similar mechanism did not function on antigen-specific TCRs. Considering that part of the TCR ligand is comprised of a self MHC molecule, an increase in affinity could result in autoreactive T cells. Thus, selection for all germline gene elements in T cell responses might be a necessary mechanism for generating

diversity in a system that does not appear to rely on somatic mutation to alter fine specificity. Whether these distinctions between TCR and immunoglobulin diversity are true in general await future studies in other antigen-specific T cell responses.

Summary

17 T cell clones and 3 T cell lines, specific for pigeon cytochrome *c*, were analyzed for fine specificity and rearranged T cell receptor (TCR) gene elements. Clones of similar fine specificities were grouped into one of four phenotypes, and correlations between phenotype differences and gene usage could be made. All the lines and clones rearranged a member of the V_α2B4 gene family to a limited number of J_α regions. The β chain was made up of one of three non-crosshybridizing V_β regions, each rearranging to only one or two J_βs. The use of alternate V_β regions could be correlated with phenotype differences, which were manifested either as MHC- or MHC and antigen–specificity changes. In addition, the presence of alloreactivity, which defined a phenotype difference, could be correlated solely with the use of an alternate J_α region. These observations were substantiated by prospective analyses of pigeon cytochrome *c*–specific T cell lines that were selected for alternate MHC specificity or alloreactivity and were found to express the correlated α and β chain rearrangements. Previously (16), the TCR DNA sequences from two clones, each representing a variant of one phenotype, showed sequence differences only in the N regions of their TCR genes. Since only these two variants, using identical V_α-J_α and V_β-J_β gene elements, were repeatedly observed in this study, we would predict that the junctional diversity differences are selectable. In this T cell response, all the gene elements involved in the generation of diversity appear to be selected, and may therefore be important in the determination of TCR specificity. This high degree of receptor gene selection represents a fundamental difference from the diversity seen in several extensively analyzed antibody responses.

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