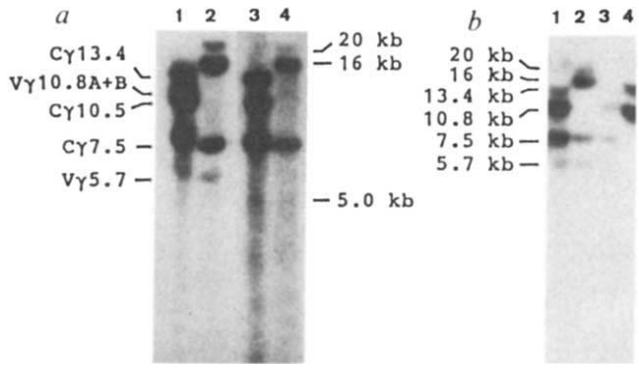

Fig. 3 Southern blot analysis of genomic and 3F9 DNA. *a*, Lanes 1 and 2 probed with ^{32}P-labelled 3F9-γ4, lanes 3 and 4 with 3F9-γ6. BALB/c kidney (lanes 1, 3), 3F9 (lanes 2, 4). *b*, BALB/c kidney, 3F9, C57BL/6 and C57BL/10 kidney DNA (lanes 1-4, respectively) probed with ^{32}P-labelled 3F9-γ4. DNA was extracted from either kidney or 3F9 T cells, digested with *Eco*RI, electrophoresed through 0.5% (*a*) or 0.7% (*b*) agarose gels, treated with 0.25 M HCl and transferred to nitrocellulose[23]. The filters were hybridized to ^{32}P-labelled probes, washed in 0.3 × SSPE, 0.1% SDS at 65 °C and autoradiographed. Each Southern blot analysis was performed five times.

cells use the same recognition mechanism via the αβ T-cell receptor, then the γ-chains are not relevant for recognition. If, however, the still elusive γ-chains are somehow involved in MHC class I recognition in antigen-specific, MHC-restricted cytotoxic T cells, then the recognition mechanism of alloreactive versus antigen-specific, MHC-restricted cytotoxic T cells is different. This could imply that alloreactive cytotoxic T cells either do not need a second receptor for recognition and mediation of effector function or that the determinants functioning as 'restriction' elements of these T cells are recognized by some other unidentified molecules.

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Note added in proof: Iwamoto *et al.* (*J. exp. Med.*, in the press) have isolated a cDNA clone from a hapten-specific T cell bearing a known V_γ region associated with a new *J-C* region which is similar to, but does not crosshybridize with, any known C_γ region genes. We have isolated similar γ-like transcripts from 3F9, but do not yet know if these clones contain an open reading frame.

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A functional γ gene formed from known γ-gene segments is not necessary for antigen-specific responses of murine cytotoxic T lymphocytes

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Structural similarities between surface immunoglobulins (s Ig) on B cells and antigen-specific receptors on T cells suggest that a T cell, like a B cell, should express only two immunoglobulin-like genes, one for each subunit of the disulphide-linked, heterodimeric, antigen-specific (αβ) T-cell receptor. However, cytotoxic T lymphocytes (T_c cells) and immature thymocytes also contain RNA transcripts of a third immunoglobulin-like gene, called γ (refs 1–4). A polypeptide corresponding to the γ gene has not yet been identified and the function of this gene remains an enigma. Judging from its nucleotide sequence, the rearranged γ gene is expected to encode an integral membrane polypeptide chain, and γ complementary DNAs from two cloned T_c cell lines have previously been found to have different sequences around the *V–J* (variable region–joining region) junction[1,2], suggesting that, in these cells, the γ-gene product is a clonally diverse surface structure that may form part of an as yet unidentified, antigen-specific receptor. To analyse further the extent of diversity of the γ-gene product, we have determined the partial sequences of 11 γ cDNA clones from three other cloned T_c cell lines, and report here that the sequences are indeed clonally diverse, but in all instances they are out-of-phase in the region of the *V–J* junction. This finding and the pattern of γ-gene rearrangements in these cell lines indicate that a polypeptide product of the previously reported γ gene, *V2J2–C2*, is not expressed in them and is, therefore, not necessary for the antigen-specific cytotoxic and proliferative responses of these mature T cells.

The cloned T_c cell lines examined (described in Table 1) were derived from BALB.B ($H\text{-}2^b$) and BALB.K ($H\text{-}2^k$) mice that had been injected several times ('hyperimmunized') with P815 cells (a DBA/2 ($H\text{-}2^d$) mastocytoma). The cell lines were specific for the class I histocompatibility antigen L^d or D^d and had the L3T4$^-$, Lyt 2$^+$ phenotype expected of T_c cells. cDNA libraries were prepared from these cell lines, γ-positive cDNA clones were identified, and partial nucleotide sequences were determined (see Fig. 1).

Hayday *et al.*[5] previously described three *V*, and *J* and three *C* (constant) γ-gene segments and demonstrated that the J_γ and C_γ gene segments are paired as in the immunoglobulin λ gene family[6,7]. To simplify discussion, these γ-gene segments are referred to here as follows (their previous designations, based on the size, in kilobases (kb), of the *Eco*RI fragment on which each is found, are given in parentheses): *V*1 (10.8B), *V*2 (10.8A), *V*3 (5.7), *J*1–*C*1 (13.4), *J*2–*C*2 (10.5) and *J*3–*C*3 (7.5).

Except for variations in the region of the *V–J* junction, the sequences of the 11 cDNA clones were all identical to the previously described sequence of γcDNA clones (pHDS 4/203)[1] from the T_c cell line 2C (also of BALB.B origin and specific for L^d)[8]. As shown in Fig. 1, in clone pHDS 4/203 from 2C cells, the insertion of an A·T dinucleotide between the *V*2 and *J*2–*C*2 gene segments established an in-frame join; the resulting sequence can encode a full-length γ polypeptide chain[1,2]. However, for all the γ cDNA clones from the three cell lines listed in Table 1, the various sequences in the vicinity of the *V-J* junction result in out-of-frame joins that are not expected to yield full-length γ chains. Thus, for γ cDNA from 3H-2 cells

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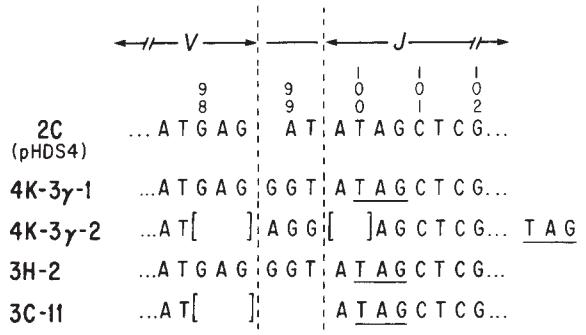

Fig. 1 Nucleotide sequences of γ cDNA clones in the region of the *V–J* junction. Nucleotides shown between the vertical broken lines are not encoded by known V_γ or J_γ gene segments[5]. Brackets indicate deletions. The codons are numbered according to the previously described γ clone pHDS4 from the T_c line 2C[1,2], and premature termination codons are underlined (the termination codon for 4K-3γ-2 occurs ~45 nucleotides downstream).
Methods. cDNA libraries were prepared in λgt10 essentially as described elsewhere[15], and screened with a C_γ probe (pHDS4)[1]. For every 100,000 plaques screened, 3–4 C_γ-hybridizing plaques and 60 C_β-hybridizing plaques were detected, on average. A total of 11 γ cDNA were sequenced by the method of Maxam and Gilbert[16]: 4 of the 4K-3γ-1 type, 2 of the 4K-3γ-2 type (both from 4K-3E), 4 of the 3H-2 type, and 1 of the 3C-11 type.

and for one of the γ cDNA clones from 4K-3E cells (4K-3γ-1), an insert of three nucleotides (GGT) at the *V–J* junction results in premature chain termination. In another γ clone from 4K-3E cells (4K-3γ-2), a different set of three nucleotides (AGG) is inserted at the *V–J* junction and both the last three nucleotides of the *V2* gene segment (GAG) and the first two nucleotides of the *J2* gene segment (AT) are deleted; the result is a termination codon 45 nucleotides downstream of the *V–J* junction. Finally, in the γ cDNA clones of 3C-11 cells, no nucleotides are inserted between the 3′ end of *V2* and the 5′ end of *J2* gene segments and there is a deletion of the last three nucleotides of *V2*; the result is a termination codon in the *J* region (Fig. 1).

The significance of the abberrant γ transcripts in the cell lines listed in Table 1 is apparent on inspection of their respective γ-gene rearrangements. As shown in the Southern blot hybridization patterns (Fig. 2), the 4K-3E cell line, like the previously described 2C cell line[2], has both the 16- and the 22-kb rearranged γ-positive fragments. In 2C cells the 16-kb fragment was shown to contain a rearranged γ gene corresponding to the assembly of *V2J2–C2* gene segments[5]. As there are no differences between the 341 sequenced nucleotides of 4K-3γ-1 (data not shown) and the *V2J2–C2* γ gene of 2C (except at the region of the *V–J* junction), it is probable that this 4K-3E γ cDNA clone represents a *V2J2–C2* γ gene on the 16-kb fragment. Indeed, there are probably two rearranged *V2J2–C2* γ genes in the 4K-3E cell line (one from each chromosome), because these cells do not have the germline configuration of the *J2–C2* γ-gene segments (Fig. 2, lane 5). It is thus of interest that a second γ cDNA clone (4K-3γ-2) from 4K-3E cells also has an out-of-frame *V–J* join (Fig. 1). The rearrangements of some of the other known gene segments also could not encode a γ-chain in these cells: thus, the third C_γ gene segment (*C3*) is defective[5], and a γ gene resulting from rearrangement of V_3 to *J1–C1* or to *J2–C2* would have been detected in Fig. 2 (see ref. 5). The 22 kb rearranged fragment in 4K-3 cells will be discussed below.

Analyses of two other cell lines, 3H-2 and 3C-11, also suggested that a polypeptide chain encoded by the *V2J2–C2* γ gene[2,5] need not be expressed in cytolytically active T_c cells. Southern blots revealed that each of these cell lines has a 16-kb,

Table 1 Cloned cytotoxic T cells

Clone	Strain of origin	Specificity	Lyt 2	L3T4
3C-11	BALB.B ($H\text{-}2^b$)	D^d	+	−
3H-2	BALB.K ($H\text{-}2^k$)	D^d	+	−
4K-3	BALB.K ($H\text{-}2^k$)	L^d	+	−

BALB.B ($H\text{-}2^b$) or BALB.K ($H\text{-}2^k$) mice were injected intraperitoneally with ~2.5×10^7 irradiated (3,000 rad) P815 cells (a DBA/2 ($H\text{-}2^d$) mastocytoma) at 3-week intervals, either three (clones 3C-11 and 3H-2) or four (clone 4K-3) times; 1–2 weeks after the final injection, alloreactive cloned T_c lines were derived from spleen cells as described elsewhere . Specificity in target cell lysis was determined by a standard 4-h ^{51}Cr-release assay[14] using as targets mouse L-cells transfected with D^d (cell line T4.8.3) or L^d (cell line T1.1.1) class I MHC antigens (provided by J. Seidman). At an effector/target cell (ratio of 5:1, the per cent ^{51}Cr-release from the appropriately labelled target cell was 58%, 83% and 39% for lines 3C-11, 3H-2 and 4K-3, respectively. The L3T4$^-$, Lyt 2$^+$ surface phenotype was determined by fluorescence-activated cell sorting (System 50-H, Ortho Diagnostic) using rat monoclonal antibodies against Lyt 2 (536.7; given by F. Fitch) and L3T4 (GK1.5; given by K. Wall).

fragment with a rearranged γ gene (Fig. 2). Moreover, the blots showed that these cells also contain unrearranged germline C_γ gene segments (compare lanes 1–4 of Fig. 2; see ref. 5). Thus it seems that in both the 3H-2 and 3C-11 cell lines there is only a single rearranged *V2J2–C2* γ gene in the γ-positive 16-kb fragment, and in both cell lines this gene is not functional. Thus, four γ cDNA clones from 3H-2 cells yielded the same sequence, which was identical to that of pHDS 4/203 of 2C cells except for the out-of-frame join in the region of the *V–J* junction (Fig. 1). For the 3C-11 cell line only a single γ cDNA clone was analysed and it also had an out-of-frame *V–J* join (Fig. 1). As these cell lines have been found (S. Latt, personal communication) to be predominantly diploid (80–85% of the cells in the 3C-11 clone and 65% of those in 4K-3L are diploid), these results suggest strongly that all of their rearranged *V2J2–C2* γ genes have out-of-frame joins.

Taking all the above results together, all three of the cytolytically active T_c cell lines studied here appear to be incapable of producing a γ-chain that is encoded by the *V2J2–C2* γ gene. Some additional V_γ gene segments, not cross-hybridizing with the *V1*, *V2* and *V3* γ-gene segments, have recently been identified (J. Heilig and S.T., manuscript submitted and ref. 18) and found, when rearranged, to be joined with the *J1–C1* segment; hence they would have been detected by screening libraries with probes that hybridize with the $C_\gamma1$ gene segment. Moreover, since both of the *J2–C2* gene segments in 4K-3 cells are rearranged to *V2* in the 16-kb fragment, it seems unlikely that the newly identified V_γ gene segments (or any additional ones) can be expressed in these cells, unless they are rearranged to as yet unidentified *J–C* γ-gene segments; if such gene segments exist, they do not cross-hybridize with the known *J–C* γ-gene segments. The arguments applied here to some cloned T_c cells also apply to certain T-helper (T_H) cells, which do not express the previously described γ-gene segments[9]. Of course, the conclusion that functional T_c and T_h cells need not express γ-chains must be considered provisional for both cell types, because it is possible that as yet unidentified V_γ and J_γ–C_γ gene segments, not cross-hybridizing with the known γ-gene segments, might encode γ-chains in these cells.

Could 4K-3 cells have in-frame γ transcript of a rearranged γ gene of the 22 kb fragment? This fragment has recently been shown to represent an incomplete *Eco*RI digest of a rearrangement in which the newly identified Vγ4 gene segment is joined to the J1-C1 gene segment (J. Heilig and S.T., in preparation). Complete digestion yielded a somewhat smaller fragment not easily resolved from the 16 kb (*V2J2–C2*) fragment. Thus, complete digestion of the *V J1–C1* γ gene may account for the

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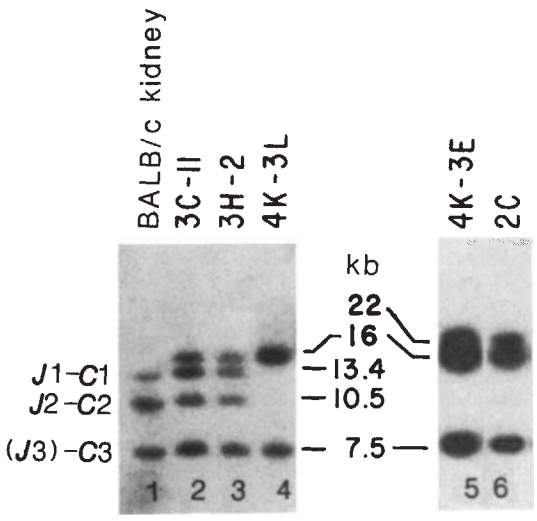

Fig. 2 Southern blot analysis of T_c DNA hybridized to a C_γ probe (pHDS4)[1]. DNA from BALB/c kidney (lane 1) has germline *J1-C1*, *J2-C2* and *(J3)-C3* gene segments on fragments of 13.4, 10.5 and 7.5 kb, respectively. DNAs from T_c lines 3C-11 and 3H-2 (lanes 2 and 3) have germline (that is, unrearranged) 13.4-kb (*J1-C1*) and 10.5-kb (*J2-C2*) fragments. The Southern blot of DNA from the 4K-3 cells (4K-3E) was repeated from cells that had been in culture for about 6 months (4K-3L). The only difference was the presence of a 22 kb fragment in the DNA from 4K-3E cells. This fragment represents an incomplete digest of a *V4J1-C1* γ gene rearrangement. The 4K-3L cells retained specificity for the L^d antigen and were virtually as active cytolytically as 4K-3E cells. DNAs (2-3 μg of each) were digested with *Eco*RI, electrophoresed through 0.8% agarose gels and blotted onto nitrocellulose[17]. Blots were hybridized at 42 °C in 50% formamide and 5 × SSC and filters were washed at 65 °C in 0.2 × SSC.

absence of the 22 kb fragment in the Southern blot of 4K-3 cells that had been in continuous culture for about six months (designated 4K-3L cells, Fig. 2, lane 4). In any case, the Vγ4 gene segment does not seem to be transcribed in 4K-3 cells or in any of the other mature T cell lines analysed thus far (unpublished observations).

The relative abundance of γ transcripts in immature thymocytes has prompted the speculation that γ-containing, antigen-specific receptors of these cells may be involved in the intrathymic selection of self-MHC (major histocompatibility complex)-restricted T cells, that is, lymphocytes that can distinguish between self- and non-self-MHC[3,4]. This distinction underlies the MHC-restriction of antigen recognition that is characteristic of T_c (and T_h) cells. There are no reasons to doubt that the three cell lines analysed here, although alloreactive (anti-L^d or D^d), are representative of typical MHC-restricted T_c cells. Whether they have ever expressed a functional γ transcript is thus a question of considerable interest. It seems unlikely that the *V2J2-C2* γ gene could have been expressed at any time in 4K-3, 3H-2 or 3C-11 cells because in these cells all copies of this gene are out-of-phase. However it is possible that a functional γ gene could have been expressed in immature progenitors of the cloned 4K-3, 3C-11, and 3H-2 cell lines formed by rearrangement of one of the recently identified *V*γ gene segments to *J1-C1* (J. Heilig and S.T., manuscript in preparation and ref. 18).

Finally, we note that altogether in the four distinct γ cDNA clones analysed here, more than 500 V_γ and J_γ nucleotides were sequenced and, except for those at the *V-J* junction, all are identical to those in the germline *V2* and *J2* gene segments[5]. The absence of nucleotide replacements is notable because the γ cDNA clones analysed were from cloned T_c cell lines that had been derived from hyperimmunized mice. In contrast, a previous analysis of immunoglobulin light-chains (λ1) transcripts from hyperimmunized mice revealed that *V*-domain nucleotide substitutions in these chains occurred with an average frequency of ~0.01 (that is ~3 per 330 nucleotides; ref. 10 and K. Tamoto, E. B. R. T. Azuma and H.N.E., in preparation). If somatic mutations in the transcribed immunoglobulin-like genes in T cells from hyperimmunized mice matched in frequency those in immunoglobulin light chain (λ1) genes in such mice, five nucleotide replacements would have been expected in the γ sequences determined here. As none were seen, our findings support growing evidence that somatic mutations are less frequent in T than in B cells[11,12]. One objection which could be raised is that the absence of somatic mutations in a rearranged γ gene that is not expressed as a polypeptide chain is not relevant for the frequency of somatic mutations in α and β genes, which are expressed as polypeptide chains. However, somatic mutations in a rearranged out-of-phase κ gene have been described[13]. Moreover, it seems reasonable to expect that cells in which somatic mutations have occurred in immunoglobulin and immunoglobulin-like genes that are expressed as polypeptide chains may well also have mutations in rearranged immunoglobulin-like genes that are transcribed but not translated into protein. Indeed, the latter mutations should be particularly informative, because they are not subject to selection by antigen or other agents (for example anti-idiotypes).

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Note added in proof: Recently another *J*γ*C*γ gene segment (J4-C4) which does not crosshybridize with the previously reported JC segments has been described by Iwamoto *et al.* (*J. exp. Med.*, in the press).

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