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# Expression of the T-cell receptor $\gamma$ -chain gene products on the surface of peripheral T cells and T-cell blasts generated by allogeneic mixed lymphocyte reaction

( $\gamma\delta$  heterodimer/anti- $\gamma$  antibody/T-cell receptor)

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**ABSTRACT** The  $\gamma$ -chain genes of the T-cell receptors form a family of related genes that are specifically expressed and somatically rearranged in T cells. Using poly- and monoclonal anti- $\gamma$  antibodies, we studied the cell-surface expression of the  $\gamma$ -chain gene products in mouse peripheral T cells as well as in the T-cell blasts generated by allogeneic mixed lymphocyte reactions. The  $\gamma$  chains are expressed in the  $Lyt2^-$ ,  $L3T4^-$  subsets of these T-cell populations as disulfide-linked heterodimers. Whereas the electrophoretic mobility and the N-glycosylation of the spleen and lymph-node  $\gamma$  chains are indistinguishable from those of the reported thymocyte  $\gamma$  chain, a minor fraction of the T blasts generated by allogeneic stimulation of B10 lymph-node T cells with B10.BR spleen cells seems to express  $\gamma$  chains with distinct properties. This suggests that the mixed lymphocyte culture conditions exert a selective effect on the expression of  $\gamma$  chains among peripheral T-cell populations.

During the search for the cDNA clones coding for the  $\alpha$  and  $\beta$  subunits of the antigen-specific T-cell receptor, we encountered a third type of gene encoding the  $\gamma$  chain that also rearranges during T-cell differentiation and exhibits striking similarities to the  $\alpha$ - and  $\beta$ -chain genes both in the primary sequence and the sequence organization (1, 2). Like immunoglobulin and T-cell receptor genes, the  $\gamma$ -chain gene is assembled by somatic recombination from variable (V), joining (J), and constant (C) gene segments (3) but the pool size of the V region of the  $\gamma$  chain gene segments is relatively small compared to those of immunoglobulin or of T-cell receptor genes (2, 4-7). In addition most (but not all) functional T cells bearing the  $\alpha\beta$  T-cell receptor harbor no functionally rearranged  $\gamma$ -chain genes (5, 6, 8, 26). Such results led to the conclusion that the  $\gamma$ -chain gene product is not essential for the functioning of conventional T cells.

Expression of the  $\gamma$ -chain gene both at the RNA and protein levels supports the above conclusion. The  $\gamma$ -chain transcripts were shown to be present primarily in the fetal and adult thymocytes exhibiting the surface phenotype characteristic of immature T cells [i.e.,  $L3T4^-$ ,  $Lyt2^-$ , hereafter referred to as double-negative (DN) cells] (9, 10) but are scarce or undetectable in the conventional mature T cells (i.e.,  $L3T4^+$ ,  $Lyt2^-$  or  $L3T4^-$ ,  $Lyt2^+$  T cells) (4, 7, 9). The first indication of the occurrence of a  $\gamma$ -chain polypeptide came from the analysis of human T cells: the  $\gamma$  chain was found to be associated with another polypeptide termed the  $\delta$  chain on the surface of a small subpopulation of peripheral T cells as well as on the surface of a thymocyte line (11, 12).

The  $\gamma$  chain was also found as a  $\gamma\delta$  heterodimer on the surface of DN thymocytes of fetal and adult mice (13-15). Subsequently, some human T-cell lines with natural killer activity were also shown to bear the  $\gamma\delta$  heterodimers (16, 17). Like the  $\alpha\beta$  heterodimer, the  $\gamma\delta$  heterodimer is associated with the monomorphic T3 protein in human and mouse (11, 14).

To the best of our knowledge, neither the function of the  $\gamma\delta$ -bearing cells nor the role of the heterodimer on these cells is known. To resolve these problems we prepared both polyclonal and monoclonal anti-mouse  $\gamma$ -chain antibodies and examined the expression of the  $\gamma\delta$  heterodimer on the surface of mouse peripheral T cells before and after *in vitro* selection by way of mixed lymphocyte reaction (MLR). We found that the  $\gamma\delta$  heterodimers are expressed on the surface of DN peripheral T cells and that the MLR conditions lead to the detection of electrophoretically distinct  $\gamma\delta$  heterodimers.

## MATERIALS AND METHODS

**Animals.** C57BL/10 (B10) and B10.BR mice were purchased from The Jackson Laboratory. BALB/c mice were purchased from the Charles River Breeding Laboratories. BALB.B and BALB.K mice were bred in the colony of the National Jewish Center for Immunology and Respiratory Medicine.

**Antisera and Monoclonal Antibodies.** Rabbit anti- $\gamma$  antibody was described (13). The production of the mouse monoclonal anti- $\gamma$  antibody will be described elsewhere. Other monoclonal antibodies used in this study are 56.3.7 (anti-Lyt-1 from American Type Culture Collection), RL172.4 (anti-L3T4 from H. R. MacDonald by way of Osami Kanagawa, Lilly Research Laboratories, La Jolla, CA), 3.155.D14 (anti-Lyt-2 from F. W. Fitch, University of Chicago) and BP107 (anti-Ia from Theresa Imanishi-Kari, Tufts University, Boston; ref. 18).

**Preparation of Cells.** The *in vitro* mixed lymphocyte reaction was carried out as described (19, 20). The fraction of T cells that are DN from various populations was prepared as follows. Thymocytes, lymph-node cells, nylon wool column-purified spleen T cells, and MLR blasts were treated with a mixture of anti-L3T4, anti-Lyt-2, and anti-Ia antibodies plus rabbit complement (Cederlane Laboratories, Hornby, ON). In most experiments this process was repeated, and viable cells were isolated on a Ficoll/Isopaque gradient.

**Cell-Surface Labeling, Immunoprecipitation, and N-Glycosidase Treatment.** Cell-surface radioiodination was performed as described (13). For immunoprecipitation after reduction-alkylation, we followed the method described (11).

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Abbreviations: V, J, and C, variable, joining, and constant regions, respectively, of T-cell receptor genes; DN, double negative; MLR, mixed lymphocyte reaction.

Briefly, the lysates were denatured by adding NaDodSO<sub>4</sub> and dithiothreitol and were alkylated by adding iodoacetamide. For immunoprecipitation under nonreducing condition, the native lysates were denatured by adding NaDodSO<sub>4</sub> but not dithiothreitol. In both cases four volumes of 1.5% (vol/vol) Triton X-100 in 10 mM Tris-buffered saline (pH 8.0) was added before immunoprecipitation. Immunoprecipitation was performed by adding rabbit anti- $\gamma$  antibody plus protein A-coupled Sepharose beads (Pharmacia) (see Figs. 2 A, B, and C, and 4B) or by adding protein A-coupled Sepharose beads precoated with monoclonal anti- $\gamma$  culture supernatant (see Figs. 1, 2D, and 4A). Solubilized precipitates were electrophoresed in 12% polyacrylamide gels. For two-dimensional PAGE, solubilized precipitates were electrophoresed in 10% polyacrylamide gel under nonreducing condition. The gel was soaked in a buffer containing 5% (vol/vol) 2-mercaptoethanol and layered on top of a 12% polyacrylamide slab gel. Peptide *N*-glycosidase F (*N*-glycosidase, Genzyme, Boston) treatment, which removes *N*-linked carbohydrate moieties, was performed as described (21).

## RESULTS

**Expression of the  $\gamma\delta$  Heterodimer on the Surface of BALB/c L3T4<sup>-</sup>,Lyt2<sup>-</sup> (DN) Splenic and Thymic T Cells.** Studies from this and other laboratories established that the murine DN thymocytes of both fetal and adult origins express a 35-kDa  $\gamma$  chain disulfide-linked to a 45-kDa  $\delta$  chain on their surface (13–15). To determine whether peripheral T cells also express  $\gamma$  chains, we prepared Nonidet P-40 lysates from surface-iodinated, DN splenic BALB/c T cells and analyzed the lysates by immunoprecipitation with anti- $\gamma$  antibodies followed by NaDodSO<sub>4</sub>/PAGE under reducing conditions. For comparison we also analyzed lysates similarly prepared from DN thymocytes. As shown in Fig. 1, lanes 2 and 5, the splenic T-cell lysates gave a 35-kDa component that comigrates with the identified  $\gamma$  chain detectable in the thymocyte lysates. The 45-kDa  $\delta$  chain is not as obvious as the  $\gamma$  chain in either lysate because of the relatively high background (lanes 1 and 6) in this size range (see below for the evidence of  $\delta$  chains in peripheral T cells).

As reported (14) and confirmed here (Fig. 1, lane 3) the apparent molecular size of the 35-kDa  $\gamma$  chain of the thymocyte origin is reduced to 33 kDa upon a treatment with the *N*-glycosidase. A similar reduction of molecular size was observed for the splenic T-cell  $\gamma$  chains when the spleen-

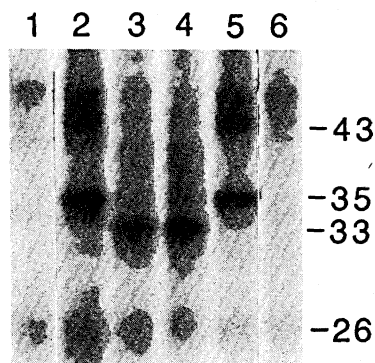


FIG. 1. Immunoprecipitation of BALB/c adult DN thymocytes and spleen cells with monoclonal anti- $\gamma$  antibody. BALB/c adult DN thymocytes (lanes 1–3) and BALB/c DN spleen T cells (lanes 4–6) were surface labeled with <sup>125</sup>I, lysed with Nonidet P-40, and immunoprecipitated with protein A-coupled Sepharose beads precoated either with monoclonal anti- $\gamma$  (lanes 2–5) or with culture medium only (lanes 1 and 6). *N*-Glycosidase treatment was done for the samples in lanes 3 and 4. In this and other figures molecular sizes are indicated in kDa.

derived lysate was subjected to the same treatment (Fig. 1, lane 4). Thus, thymic and splenic  $\gamma$  chains are both *N*-glycosylated and their molecular sizes are indistinguishable by PAGE analysis both before and after removal of the *N*-linked carbohydrates.

**The  $\gamma$  Chain from B10 Mice Lacks *N*-Glycosylation.** Since B10 mice have a variety of H-2 congenic strains and are, therefore, potentially useful in the search for the putative ligand for the  $\gamma\delta$  heterodimer, we examined the heterodimer on the surface of B10 thymocytes and peripheral T cells. Fig. 2 A and B shows the “off-diagonal” PAGE analysis of the immunoprecipitates of lysates prepared from surface-iodinated B10 spleen T cells and lymph-node cells, respectively. A pair of “off-diagonal” spots reminiscent of the reported

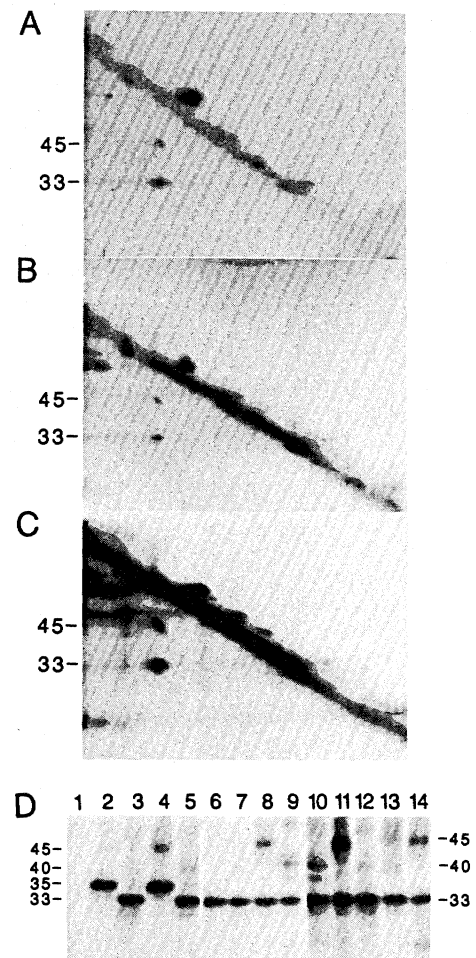


FIG. 2. Immunoprecipitation of B10 peripheral lymphocytes and thymocytes by anti- $\gamma$  antibody. (A–C) “Off-diagonal PAGE” analysis. Surface radiiodinated B10 spleen T cells (A), B10 lymph-node cells (B), and B10 DN lymph-node cells (C) were immunoprecipitated with rabbit anti- $\gamma$  antibody. PAGE was performed under nonreducing conditions from left to right and reducing conditions from top to bottom. (D) Analysis by one-dimensional PAGE with and without a prior *N*-glycosidase treatment. BALB/c DN thymocytes (lanes 1–5), B10 DN thymocytes (lanes 6–9 and 14), and B10 DN spleen T cells (lanes 10–13) were surface-labeled with <sup>125</sup>I, lysed with Nonidet P-40, and immunoprecipitated either under nonreducing conditions (lanes 1, 4, 5, 8, 9, 10, 11, and 14) or after prior reduction and alkylation (lanes 2, 3, 6, 7, 12, and 13). Immunoprecipitation was carried out by adding protein A-coupled Sepharose beads either precoated with the monoclonal anti- $\gamma$  culture supernatant (lanes 2–14) or preincubated with culture medium (lane 1). *N*-Glycosidase treatment was performed for the samples analyzed in lanes 3, 5, 7, 9, 10, and 12. Prior to applying to the gel, all precipitates were reduced by being boiled for 5 min in the presence of 5% (vol/vol) 2-mercaptoethanol.

thymic  $\gamma\delta$  heterodimers (13–15) are present in the spleen and lymph-node T-cell lysates. Furthermore, most of these heterodimers are present on the surface of the DN population of lymph-node (Fig. 2C) and spleen T cells (data not shown).

The apparent molecular size of the B10  $\delta$  chain is 45 kDa as is the  $\delta$  chain of BALB/c (13) and C57BL/6 thymocytes (14, 15). However, the B10  $\gamma$  chain seems to migrate significantly faster than the  $\gamma$  chains of BALB/c or C57BL/6 origins; the estimated molecular size of the B10  $\gamma$  chain is 33 kDa, whereas BALB/c (see Fig. 1 and ref. 13) and C57BL/6  $\gamma$  chains are 35 kDa. The one-dimensional PAGE analysis illustrated in Fig. 2D confirms that the  $\gamma$  chain of BALB/c thymocytes (35 kDa, lane 4) is indeed greater than the  $\gamma$  chain of B10 spleen T cells (33 kDa, lane 11). In contrast to the BALB/c  $\gamma$  chain (lanes 4 and 5), the B10  $\gamma$  chain is not detectably altered by the *N*-glycosidase treatment (lanes 10 and 11) and comigrates with the de-*N*-glycosylated BALB/c  $\gamma$  chain. Furthermore the  $\gamma$  chain of B10 thymocytes (lanes 8, 9, and 14) is indistinguishable from that of B10 spleen cells in the mobility and *N*-glycosidase resistance. These results strongly suggest that there occurs a polymorphism in the  $\gamma$ -chain gene(s) between BALB/c and B10 strains such that the N-linked glycosylation site present in the former strain is missing in the latter. In addition the results presented in Fig. 2D demonstrate that the 33- or 35-kDa component is indeed the  $\gamma$  chain (lanes 2, 3, 6, 7, 12, and 13) and that the  $\delta$  chain is about 45 and 40 kDa before and after the *N*-glycosidase treatment, respectively (lanes 4, 5, 8, 9, 10, and 11).

**The  $\gamma\delta$  Heterodimer Is Expressed on the Surface of Lyt-2<sup>-</sup>,L3T4<sup>-</sup> T Cells Grown in Allogeneic Mixed Lymphocyte Cultures.** Because of the striking structural similarity of the

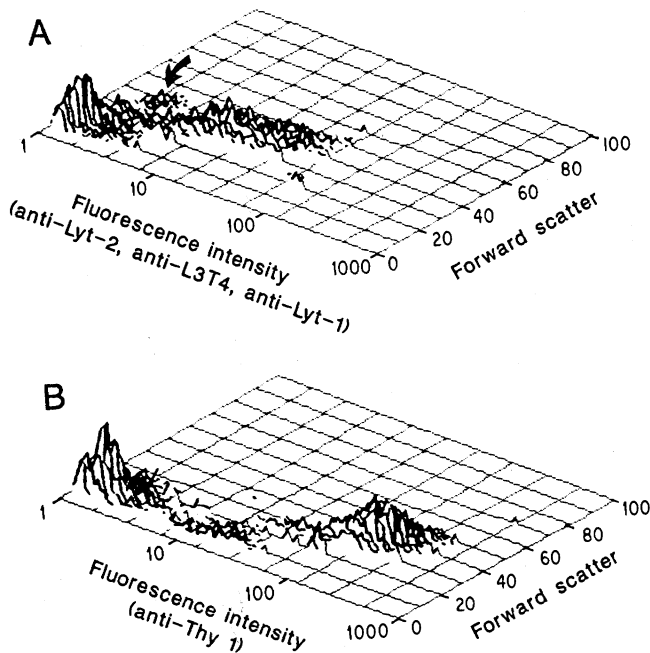


FIG. 3. Cytofluorimetric analysis of *in vitro* day 5 MLR blasts. (A) MLR blasts (BALB.B anti-BALB.K) were incubated first with a mixture of rat monoclonal antibodies (anti-Lyt-1, anti-Lyt-2, and anti-L3T4) and subsequently with fluorescein isothiocyanate-labeled MAR.18 (mouse anti-rat immunoglobulin  $\kappa$ ). The arrow indicates MLR blasts that display the Lyt-1<sup>-</sup>,Lyt-2<sup>-</sup>,L3T4<sup>-</sup> phenotype. The Ortho Cytofluorograf system 50 was used for analysis. (B) Same cells were stained with rat anti-Thy-1 antibody and fluorescein isothiocyanate-labeled MAR.18. Note that virtually all blasts (i.e., forward scatter high cells) are Thy-1<sup>+</sup>, indicating that the Lyt-1<sup>-</sup>,Lyt-2<sup>-</sup>,L3T4<sup>-</sup> cells are Thy-1<sup>+</sup>. Similar results were obtained in various responder-stimulator combinations, irrespective of their major histocompatibility complexes and/or genetic backgrounds.

$\gamma$ -chain gene to the T-cell receptor  $\alpha$ - and  $\beta$ -chain genes, it has been suggested that major histocompatibility complex products may constitute at least part of the putative ligand for the  $\gamma\delta$  receptor (4, 9, 22). To begin to investigate this possibility, we grew lymph-node T cells in an allogeneic mixed lymphocyte culture and examined the expression of  $\gamma\delta$  heterodimers on the surface of the blast cells. Thus, B10 lymph-node cells were stimulated with irradiated B10.BR spleen cells *in vivo* and restimulated *in vitro*, and then the viable cells were purified from the 5-day cultures. The precipitation of MLR-blast lysates with anti- $\gamma$  antibodies led to the detection of a typical  $\gamma\delta$  heterodimer (data not shown).

As shown in Fig. 3, a fraction of the MLR cells are Thy-1<sup>+</sup> DN blasts. We enriched these cells by complement-mediated killing, analyzed their lysates and found that most of the heterodimers were present on the surface of DN blasts (data not shown). In the next analysis the immunoprecipitates obtained with DN thymocytes, syngeneic MLR blasts, and allogeneic MLR-blast lysates were first electrophoresed separately under nonreducing conditions. Only 2-cm sections of the gels that would contain the  $\gamma\delta$  heterodimers were cut out and applied side by side to a single slab gel for the second electrophoresis under reducing conditions. With the DN thymocytes and the syngeneic MLR blasts, the major  $\gamma$  component migrated as a 33-kDa spot. By contrast the  $\gamma$  chains of the allogeneic MLR blasts appeared as a double

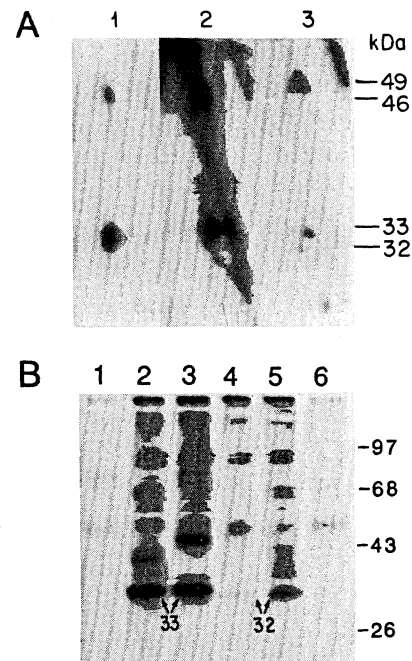


FIG. 4. Comparison of  $\gamma\delta$  heterodimers from MLR blasts and B10 thymocytes. (A) <sup>125</sup>I-surface-labeled lysates from B10 DN thymocytes (lane 1), B10 anti-B10 syngeneic MLR blasts (lane 2), and B10 anti-B10.BR allogeneic MLR blasts (lane 3) were immunoprecipitated with protein A-coupled Sepharose beads precoated with a monoclonal anti- $\gamma$  antibody and electrophoresed separately under nonreducing conditions from left to right. Portions of the gels that would contain the heterodimers were cut out, loaded side by side onto a single slab gel, and electrophoresed from top to bottom under reducing conditions. Note that the electrophoresis in the second dimension was carried out for a prolonged period (15 hr) to resolve the two  $\gamma$  components. (B) B10 DN thymocytes (lanes 1–3) and DN MLR blasts (lanes 4–6) were <sup>125</sup>I-surface-labeled, lysed with Nonidet P-40, and immunoprecipitated either with rabbit anti- $\gamma$  antibody plus protein A-coupled Sepharose beads (lanes 2–5) or with normal rabbit immunoglobulin plus protein A-coupled Sepharose beads (lanes 1 and 6). *N*-Glycosidase treatment was performed for the samples analyzed in lanes 2 and 5.

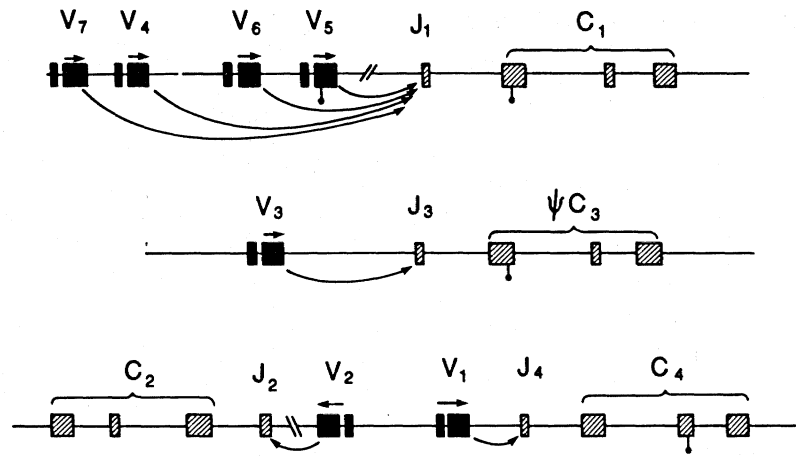


FIG. 5. Organization of mouse  $\gamma$  chain of the T-cell receptor gene segments in BALB/c germ-line genome. The map is based on refs. 3–7 and 24. Arrows indicate preferred V–J joinings.  $C_3$  is thought to be defective (3). Potential N-glycosylation sites are indicated by the symbol  $\psi$ . Double slashes indicate lack of information about the linkage.

spot of 33 and 32 kDa (Fig. 4A). Neither the 33- nor the 32-kDa  $\gamma$  chain seem to be N-glycosylated (Fig. 4B).

## DISCUSSION

Studies from this and other laboratories have demonstrated that DN mouse thymocytes of both fetal and adult origins express on their surface the product of the T-cell-specific, rearranging  $\gamma$ -chain gene as a N-glycosylated subunit of a disulfide-linked heterodimer,  $\gamma\delta$  (13, 15). The present study showed that (i) DN-peripheral (spleen and lymph-node) T cells also express the  $\gamma\delta$  heterodimers whose electrophoretic mobility and glycosylation properties are indistinguishable from those of thymocytes (ii) B10 and BALB/c mice exhibit polymorphic differences in the expressed  $\gamma$ -chain gene products, and (iii) DN T-cell blasts generated in allogeneic mixed lymphocyte cultures express  $\gamma\delta$  heterodimers that seem to be distinct from those detected on the thymocytes or syngeneic MLR blasts.

Since the discovery of the first  $\gamma$  cDNA clone pHDS4/203, a variety of germ-line BALB/c mouse  $\gamma$ -chain gene segments were cloned using this cDNA sequence as the hybridization probe either directly or indirectly (3–7, 23). The organization of these gene segments is illustrated in Fig. 5. The characteristic features of the mouse  $\gamma$ -chain gene family are (i) a high (albeit not complete) preference for some types of V–J rearrangements as indicated in Fig. 5, (ii) rearrangements involving  $C_1$  and  $C_2$  gene segments are frequent while those involving  $C_4$  are rare among thymocytes and T cells, and (iii)  $C_1$ -associated  $\gamma$ -chain genes carry potential sites for N-linked glycosylation, whereas the  $C_2$ -associated  $\gamma$ -chain gene ( $V_2J_2C_2$ ) has no such site. Because the  $\gamma$  chains expressed on thymocytes are N-glycosidase sensitive, they are thought to be primarily encoded by  $C_1$ -associated  $\gamma$ -chain genes (14). It follows by the same reasoning that the  $\gamma$  chains expressed on the DN subset of peripheral T cells are also primarily encoded by the  $C_1$ -associated  $\gamma$ -chain genes.

The most straightforward interpretation of the un-N-glycosylated  $\gamma$  chains detected on the surface of B10 thymocytes and peripheral T cells is that these  $\gamma$  chains are encoded also by  $C_1$ -associated  $\gamma$ -chain genes but these genes lack, due to polymorphism, the codons encoding amino acids that can act as a site for N-glycosylation. Unfortunately B10  $\gamma$ -chain gene segments are yet to be sequenced to confirm this hypothesis. However, we noticed the presence of multiple restriction site polymorphism specifically around the B10 and BALB/c  $C_1$  gene segments (ref. 22; and D. Kranz and S.T., unpublished results). A less-probable explanation for this

observation is that we are witnessing a strain-dependent selection of the  $\gamma$ -chain gene expressed in T cells; in this case, the 33-kDa, un-N-glycosylated B10  $\gamma$  chains would be encoded by  $\gamma$ -chain genes other than the  $C_1$ -associated genes (such as  $V_2J_2C_2$ ).

About half of the  $\gamma$  chains expressed on the surface of the allogeneic MLR blasts are 32 kDa, which is barely detectable on thymocytes or syngeneic MLR blasts. One possible interpretation of this striking observation could be that the two slightly distinct  $\gamma$  chains (32 kDa and 33 kDa) are the products of two distinct  $\gamma$  genes ( $V_2J_2C_2$  and  $C_1$ -linked  $\gamma$  genes, respectively). In agreement with this hypothesis is the observation that allogeneic MLR T cells contain full-size  $V_2J_2C_2$  gene transcripts, and the majority of them are in-frame V–J joined (25). The above interpretation is further supported by the fact that the BALB/c  $C_2$  gene segment is 5 amino acids shorter than BALB/c  $C_1$  gene segment due to an internal deletion (4). If the same applies to B10  $\gamma$ -chain genes, the difference in the length of the backbone polypeptide chain could account for the small molecular size difference between the 32- and 33-kDa  $\gamma$  chains. This interpretation implies that the allogeneic MLR culture conditions either induce  $V_2J_2C_2$  gene expression or select  $V_2J_2C_2$ -chain-bearing cells for preferential growth.

Regardless of whether the proposed gene–polypeptide chain assignment is correct or not, the allogeneic MLR conditions seem to exert a strong inductive or selective effect on the expression of  $\gamma$ -chain genes or on the cells bearing their gene products. Determining whether this effect is mediated by the recognition of the major histocompatibility complex difference by the  $\gamma\delta$  receptor would be very informative for our understanding of the recognition property and function of  $\gamma\delta$ -bearing cells.

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