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# The Nucleocapsid of Hepatitis B Virus Is Both a T-Cell–Independent and a T-Cell–Dependent Antigen

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**One characteristic of the immune response during hepatitis B virus (HBV) infection in humans is the vigorous production and subsequent persistence of antibodies of immunoglobulin (Ig) classes M and G to the nucleocapsid antigen (HBcAg). In this study HBcAg was shown to be similarly immunogenic in mice. When injected into athymic (nude) B10.BR and athymic BALB/c mice, HBcAg induced IgM and IgG class antibodies to HBc in spite of the absence of T cells in nude mice. In euthymic mice, HBcAg efficiently stimulated T-cell proliferation in vitro and helper T-cell function in vivo. The dual functions of HBcAg as a T-cell–independent and a T-cell–dependent antigen may explain its enhanced immunogenicity. Denaturation of HBcAg yields a nonparticulate antigen designated HBeAg; when HBeAg was used as the immunogen, antibody production required helper T-cell function. Although HBcAg and HBeAg are serologically distinct, they are structurally related, and in these experiments were highly cross-reactive at the T-cell level. These results suggest that the elevated levels of IgM antibodies to HBc and the enhanced immunogenicity of HBcAg during HBV infection in humans reflect the ability of HBcAg to directly activate B cells to produce antibodies to HBc in the presence or absence of HBcAg- or HBeAg-sensitized T cells.**

DURING INFECTION WITH HEPATITIS B virus (HBV), at least four antigen-antibody systems are observed: hepatitis B surface antigen (HBsAg) and its antibody (anti-HBs); the pre-S antigens associated with HBsAg particles and their antibodies; the particulate nucleocapsid antigen (HBcAg) and anti-HBc; and an antigen structurally related to HBcAg, namely, HBeAg and its antibody (anti-HBe). When HBcAg is treated with proteolytic enzymes or mild detergent it is converted to HBeAg (*1, 2*). This has been confirmed by the finding that cryptic HBeAg determinants are present on recombinant HBcAg (rHBcAg) (*3*).

The HBsAg, including the pre-S region, is an early marker of HBV infection. Although the development of antibodies to HBsAg may be delayed, antibodies to pre-S antigens may occur quite early in infection (*4*). Nevertheless, the humoral responses to the envelope antigens may vary considerably from patient to patient during infection and after vaccination, ranging from high to nonresponder phenotypes (*5*). Despite being internal components of virions, nucleocapsid antigens of a number of human viruses are efficient immunogens (for example, HTLV-I, HTLV-III, hepatitis Delta virus, and Epstein-Barr virus). The HBcAg is no exception since high titers of anti-HBc are regularly produced by virtually 100% of HBV-infected patients. Furthermore, anti-HBc of the immunoglobulin M (IgM) class appears early in acute hepatitis B, and IgM and IgG anti-HBc can persist with slowly decreasing titers for many years (*6*). In contrast, antibodies to HBeAg may not develop or may appear at various times after the appearance of anti-HBc, and are correlated with viral clearance (*7*).

Table 1. Comparison of primary antibody responses after immunization with HBsAg and HBcAg.

| Strain* | H-2 | Anti-HBs (titer) | Anti-HBc (titer) |
|---|---|---|---|
| B10 | b | 256 | 40,960 |
| B10.D2 | d | 1024 | 81,920 |
| B10.S | s | 0† | 163,840 |
| B10.BR | k | 32 | 163,840 |
| B10.M | f | 0† | 20,480 |
| $C_3H.Q$ | q | 2048 | 327,680 |
| BALB/c | d | 1024 | 327,680 |

*Groups of five mice from each strain were immunized with 4.0 μg of HBsAg or HBcAg in CFA, and pooled sera were analyzed by solid-phase RIA for IgG antibodies of the indicated specificities at day 24. Data are expressed as the reciprocal of the highest serum dilution to yield four times the counts of the sera before immunization (titer). †The H-$2^s$ and H-$2^f$ haplotypes are nonresponsive to HBsAg even after secondary immunization (*21*).

Although HBcAg and HBeAg are serologically distinct, the primary amino acid sequences show significant identity [serum HBeAg lacks the COOH-terminal 35 residues of HBcAg (*8*)]. One might therefore predict that these antigens cross-react at the T-cell level. Why then do the immune responses to HBcAg and HBeAg appear to be regulated independently? This apparent contradiction, the enhanced immunogenicity of HBcAg, and reports that vaccination with HBcAg confers protection against HBV infection prompted us to examine the murine humoral and cellular immune responses to HBcAg.

A number of inbred murine strains, including a series of H-2-congenics, were immunized with 4 μg of rHBcAg or HBsAg (both particulate antigens) in complete Freund's adjuvant (CFA), and the primary IgG antibody responses were analyzed by solid-phase radioimmunoassays (RIA) of approximately equal sensitivities. The results of these assays correlated with results obtained with commercially available anti-HBs and anti-HBc assays (Abbott), and were of equal to greater sensitivity. All strains immunized with HBcAg showed a vigorous, primary, IgG anti-HBc response (Table 1). The influence of H-2–linked genes on the anti-HBc response is apparent (B10.S, B10.BR>B10, B10.D2, B10.M), although no nonresponder strains were identified. The anti-HBc responses were significantly greater (at least 80-fold) than the anti-HBs responses in all strains tested. Furthermore, high-titered anti-HBc persisted in these mice a year after this single dose of HBcAg. The comparative magnitudes of the primary anti-HBc and anti-HBs responses, and the lack of nonresponsiveness to HBcAg are, in general, consistent with the human immune responses to these HBV antigens.

The enhanced immunogenicity of HBcAg in mice prompted us to examine the ability of HBcAg to activate B cells directly. Therefore, B10.BR euthymic (+/+) and B10.BR athymic (nu/nu) mice were immunized with various doses (0.5, 1.5, and 4.0 μg) of rHBcAg in CFA, and sera were analyzed for anti-HBc at 10 and 24 days after immunization. As expected, the B10.BR +/+ mice produced dose-dependent anti-HBc at 10 days and a 4- to 16-fold increase in the anti-HBc titer at 24 days (Fig. 1A). However, the B10.BR nu/nu mice also produced dose-dependent, anti-HBc antibody at 10 days after immunization (Fig. 1B), but showed no increase in the anti-HBc titer at 24 days. Complete Freund's adjuvant was not re-

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quired, since B10.BR nu/nu mice immunized with HBcAg in incomplete adjuvant also produced anti-HBc, although of lesser titer. In addition, anti-HBc production by athymic mice was not unique to *Escherichia coli*–derived rHBcAg, since immunization with yeast-derived rHBcAg elicited an equivalent response.

To determine if T-cell independence required that the HBcAg be particulate, we also immunized the B10.BR +/+ and B10.BR nu/nu strains with detergent and 2-mercaptoethanol–denatured HBcAg (D-HBcAg) (Fig. 1, A and B). Although D-HBcAg was significantly less immunogenic than native HBcAg in B10.BR +/+ mice, antibodies (reactive with denatured HBcAg) were detectable by day 24. In contrast, B10.BR nu/nu mice showed no response to D-HBcAg, indicating that the response to D-HBcAg was T cell–dependent, unlike the HBcAg-specific response (Fig. 1, A and B). Since D-HBcAg bound monoclonal antibody to HBe and expressed less than 5% of the original HBc antigenicity, D-HBcAg represented HBeAg as an antigen. However, a caveat to the assumption that D-HBcAg also represents HBeAg as an immunogen is the possibility that naturally occurring HBeAg has a different subunit structure (that is, degree of polymerization) from that of D-HBcAg.

To examine the relative kinetics of anti-HBc and anti-HBs production in vivo and to demonstrate that the HBcAg preparation possessed no inherent adjuvanticity, we immunized BALB/c euthymic (+/+) and athymic (nu/nu) mice with a mixture of HBcAg (8 μg) and HBsAg (8 μg) (Fig. 2, A and B). The BALB/c +/+ mice produced anti-HBc as early as 6 days after immunization and the titer continued to rise through day 24; anti-HBs, however, was not detected until day 12 and was of significantly lower titer throughout the observation period (Fig. 2A). In contrast, the BALB/c nu/nu mice produced no anti-HBs at all, but produced anti-HBc as early as day 6; the titer peaked at day 12 and began to decline by day 24 (Fig. 2B). The lack of an anti-HBs response in BALB nu/nu mice is consistent with the T-cell–dependent nature of HBsAg as previously described (*9*). However, it is clear from this and the previous experiment that HBcAg can function as a T-cell–independent antigen.

Table 2. Class and subclass distribution of anti-HBc produced in B10.BR euthymic (+/+) and B10.BR athymic (nu/nu) mice.

| Strain* | Time after immunization (days) | Anti-HBc (titer) | | | | | |
|---|---|---|---|---|---|---|---|
| | | IgM | PolyIgG | $IgG_1$ | $IgG_{2a}$ | $IgG_{2b}$ | $IgG_3$ |
| B10.BR +/+ | 10 | 2,560 | 40,960 | 40 | 2,560 | 10,240 | 640 |
| | 24 | 1,280 | 163,840 | 640 | 20,480 | 163,840 | 2,560 |
| B10.BR nu/nu | 10 | 1,280 | 2,560 | 0 | 0 | 2,560 | 0 |
| | 24 | 1,280 | 2,560 | 0 | 40 | 2,560 | 0 |

*Groups of five euthymic (+/+) or athymic (nu/nu) mice were immunized with 4.0 μg of HBcAg in CFA, and sera were analyzed by RIA for antibodies to HBcAg of the IgM class and IgG class and subclasses (using IgG subclass-specific second antibodies) at days 10 and 24. Data are expressed as the reciprocal of the highest serum dilution to yield four times the counts of sera before immunization (titer).

We next examined the possibility that T-cell responses specific for HBcAg and D-HBcAg (that is, HBeAg) might account for the differential immunogenicity of these two antigens. High responder mice of the $C_3H.Q$ strain were immunized with 4 μg of either HBcAg or D-HBcAg. Draining lymph node cells were then harvested and challenged in vitro with both antigens, and antigen-dependent interleukin-2 (IL-2) production was measured. To ensure that the D-HBcAg used for in vitro stimulation was devoid of HBc antigenicity, we subjected the HBcAg to sonication until the antigen bound neither anti-HBc nor anti-HBe; this antigen was designated $D_s$-HBcAg. The responses of HBcAg-primed T cells to both HBcAg and $D_s$-HBcAg were equivalent at the high end of the dose response curve (0.06 to 1.0 μg/ml) (Fig. 3). The particulate form was more efficient at the low end of the

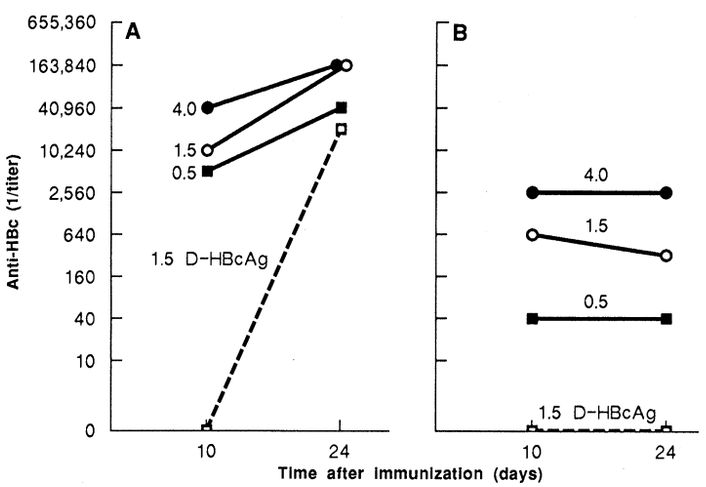


Fig. 1 (left). The HBcAg can function as a T-cell–independent antigen. Groups of five B10.BR euthymic (+/+) (A) or B10.BR athymic (nu/nu) (B) mice were immunized intraperitoneally with a single dose of the indicated amounts of *E. coli*–derived, recombinant HBcAg (0.5, 1.5, or 4.0 μg) (Biogen) or with 1.5 μg of denatured HBcAg (D-HBcAg) in CFA. HBcAg was denatured by treatment with a final concentration of 0.1% SDS and 0.1% 2-mercaptoethanol for 2 hours at 37°C. The resulting preparation was reactive with monoclonal antibodies to HBeAg, but lost greater than 95% of its reactivity with monoclonal antibodies to HBcAg. Monoclonal antibodies (*8*) were provided by M. Mayumi (Jichi Medical School, Japan). At 10 and 24 days after immunization, sera were collected, pooled, and analyzed for anti-HBc activity by solid-phase RIA. HBcAg or D-HBcAg served as the solid-phase ligand, goat antibody to mouse Ig was the second antibody, and $^{125}I$-labeled swine antibody to goat Ig was used as the probe. Data were expressed as the reciprocal of the highest serum dilution to yield four times the counts of sera before immunization.

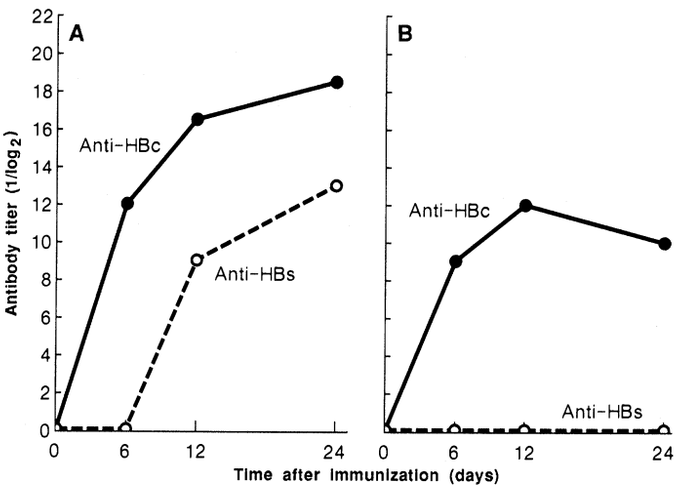


Fig. 2 (right). The production of anti-HBc in euthymic BALB/c mice precedes and is of greater magnitude than that of anti-HBs. Groups of five BALB/c euthymic (+/+) (A) or BALB/c athymic (nu/nu) (B) mice were immunized intraperitoneally with a mixture of rHBcAg (8 μg) and HBsAg (8 μg) in CFA. Serum samples obtained before and 6, 12, and 24 days after immunization were pooled and analyzed for anti-HBc activity as described in Fig. 1 and anti-HBs activity as described elsewhere (*21*). Data are expressed as the reciprocal of the $\log_2$ of the highest serum dilution to yield four times the counts of sera before immunization.

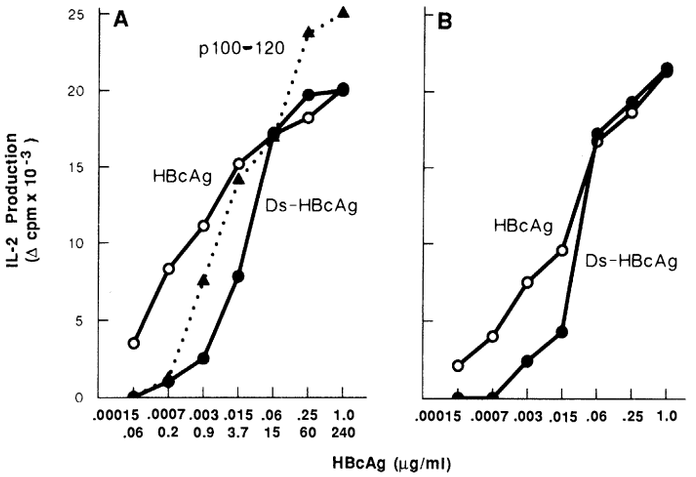


Fig. 3. HBcAg and HBeAg are cross-reactive at the T-cell level. Groups of four $C_3H.Q$ mice were primed in the hind footpads with either 4.0 μg of HBcAg (A) or 4.0 μg of denatured HBcAg (D-HBcAg) (B) in CFA. After 8 days, draining lymph node cells were harvested, pooled, and cultured with various concentrations of HBcAg, sonicated HBcAg ($D_s$-HBcAg), an HBcAg/HBeAg-specific synthetic peptide representing residues 100 to 120 (concentration; lower *x*-axis), or medium alone. The $D_s$-HBcAg was sonicated to the extent that it was totally unreactive with anti-HBc and anti-HBe monoclonal antibodies. T-cell activation was measured by antigen-induced IL-2 production. Twenty-four hours after the initiation of the in vitro cultures, supernatants were collected and assayed for IL-2 content using cells of an IL-2–dependent cell line (NK-A) (*22*). IL-2 production is expressed as [$^3$H]thymidine incorporation by NK-A cells cultured in supernatant from antigen-treated cultures minus incorporation that occurred in supernatant from control cultures not treated with antigen (ΔCPM). This is a representative assay of experiments performed on three separate occasions.

dose response curve. Note that HBcAg elicited IL-2 production by HBcAg-primed T cells at an antigen concentration as low as 0.15 ng/ml. In the reciprocal experiment, D-HBcAg–primed T cells recognized HBcAg and $D_s$-HBcAg in a manner similar to HBcAg-primed T cells (Fig. 3B). Since the HBcAg particle was recognized better, regardless of the priming antigen, the particulate form of the antigen may be more efficiently phagocytosed and presented to T cells. Furthermore, preliminary experiments have shown that unpurified, rHBeAg can activate HBcAg-primed T cells. These results indicate that HBcAg and HBeAg are virtually indistinguishable at the T-cell level even though HBcAg and HBeAg are serologically distinct. In support of this, a 21-residue synthetic peptide representing residues 100 to 120 within the HBcAg/HBeAg sequence was capable of activating HBcAg-primed T cells in vitro (Fig. 3A). In the reciprocal experiment, T cells primed with the 100–120 residue fragment were activated by native HBcAg. We have identified additional HBcAg/HBeAg peptide sequences that induce HBcAg-specific T-cell proliferation and none of them bind monoclonal antibodies to HBe or HBc. These results show that T-cell recognition of HBcAg/HBeAg differs from B-cell recognition in that T cells do not require the protein conformation necessary to preserve the antibody binding sites on either HBcAg or HBeAg. Since the production of anti-HBc and anti-HBe appears to be regulated independently during HBV infection, the cross-reactivity of T-cell recognition of HBcAg and HBeAg suggests that the differences between the antibody responses must be explained at the B-cell level.

To determine if the production of IgG class anti-HBc strictly required T-cell influence, we investigated the Ig class and IgG subclass distribution of anti-HBc production in B10.BR +/+ and B10.BR nu/nu mice immunized with HBcAg (Table 2). Euthymic B10.BR mice produced anti-HBc of the IgM class and the entire spectrum of IgG subclasses at 10 and 24 days after immunization, although antibodies of the $IgG_{2b}$ subclass predominated. Note that IgM antibody declined slightly between days 10 and 24, whereas IgG antibodies showed a 4- to 16-fold increase. The B10.BR nu/nu mice also produced an IgG class anti-HBc response; the titer was equivalent or higher than that of the IgM class antibodies; however, the IgG response was exclusively of the $IgG_{2b}$ subclass (Table 2). Therefore, production of anti-HBc of the IgG class per se is not a marker of T-cell sensitization, but increased IgG subclass diversification and elevated IgG titers are such markers. In these experiments we used a single, relatively small dose of HBcAg, whereas the amount of HBcAg produced during HBV infection would be greater, and would persist throughout the viral replicative phase.

A characteristic of HBV infection is vigorous IgM anti-HBc production early during the acute stage of infection (*10, 11*). Many chronically infected patients also maintain IgM anti-HBc, although usually at lower titers (*12*). In contrast, HBsAg elicits a relatively weak IgM response during infection and after vaccination (*13*). In a comprehensive serological study of IgM anti-HBc production during HBV infection, Gerlich *et al.* (*14*) reported variation in the kinetics of IgM persistence in resolving acute hepatitis, and a very slow decrease or even increase in IgM anti-HBc in patients progressing to chronicity. Furthermore, in patients with chronic hepatitis B in whom viral replication was reactivated by prednisone therapy, the IgM anti-HBc levels were dramatically elevated with no change in IgG titers (*15*). These findings are consistent with the notion that IgM anti-HBc production in HBV infection may be T cell–independent and, furthermore, that the switch from predominantly IgM to high titers of IgG anti-HBc requires T-cell helper function, which may be variably present from patient to patient and defective in patients who progress to chronicity. This would explain the slow decline in IgM anti-HBc titers during the first 1 to 2 years of chronic infection. Since T-cell recognition of HBcAg and HBeAg is highly cross-reactive, T-cell help for IgG anti-HBc production would presumably also help anti-HBe production. This would predict that the decline of IgM in favor of IgG anti-HBc should correlate with anti-HBe seroconversion, and such a correlation has been suggested (*14*).

These results may also be relevant to the observation that immunization with HBcAg protected chimpanzees against HBV infection (*16, 17*). Since antibodies to HBcAg coexisted with infectious HBV in serum and since transplacentally acquired anti-HBc appeared not to protect HBV-exposed newborns, it was suggested that protection may be through clearance of infected hepatocytes by HBcAg-specific T lymphocytes (*16*). Since HBcAg immunization can prime HBeAg-specific T-cell function, the mechanism of protection may have been through an HBeAg-specific cellular response.

Our results show that the particulate form of HBcAg is necessary for T cell–independent antibody production, since D-HBcAg is a T cell–dependent antigen. Antigens that are T cell–independent are generally nonprotein, polymeric molecules of relatively high molecular weight with repeating arrays of identical epitopes or haptenic determinants. The HBcAg fulfills these criteria, being a 27-nm particle composed of multiple copies of a single polypeptide (P21). In addition, the protein nature of HBcAg permits recognition by T cells as shown herein. The immunogenic effects of an antigen that has both T-cell–independent and T-cell–dependent characteristics may well be syner-

gistic in the presence of competent T cells. This property of HBcAg may explain its strong immunogenicity. However, not all antigens of this general type are T cell–independent (for example, HBsAg). It has been suggested that a T-cell–independent molecule must contain a threshold number of appropriately spaced haptens or epitopes necessary for simultaneous B-cell receptor binding (*18*). The two HBV particulate antigens HBcAg and HBsAg may differ in this respect. The nucleocapsid protein of HBV, like that of other animal viruses, possesses protein kinase activity (*19, 20*). The rHBcAg used in these experiments exhibited such activity, and the influence of this property of HBcAg on immunogenicity warrants further study. The extent to which the enzymatic, structural, and immunologic properties described herein for HBcAg are shared with the nucleocapsid antigens of other viruses remains to be determined.


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# Characterization of T Cell Receptor Gamma Chain Expression in a Subset of Murine Thymocytes


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**While much information exists about the structure and function of the clonally distributed T cell receptor (TCR) αβ heterodimer, little is known about the γ protein, the product of a third rearranging TCR gene. An antiserum to a carboxyl-terminal peptide common to several of the murine gamma chain constant regions and a monoclonal antibody to the murine T3 complex were used to identify products of this TCR gene family in a subpopulation of Lyt2$^-$, L3T4$^-$ thymocytes. This subpopulation does not express TCRα or full-length TCRβ messenger RNA. The gamma chain is a 35-kilodalton (kD) protein that is disulfide-bonded to a 45-kD partner and is associated with the T3 complex. Analysis of the glycosylation pattern of this thymic gamma chain revealed that the major variable region gamma (Vγ) gene transcribed in activated peripheral T cells is absent from this subpopulation. The cells that bear this second T cell receptor may therefore represent a distinct lineage differentiating within the thymus.**


ANTIGEN-SPECIFIC, MHC-RESTRICTED recognition by mature T cells appears to be mediated by the clonotypic αβ heterodimeric receptor (*1*). These proteins are noncovalently associated with the multicomponent T3 complex, which appears to be responsible for transduction of receptor-mediated activation signals (*2*). A search for the genes encoding the α and β proteins revealed a third T cell receptor (TCR)–like gene, termed γ, which contains variable (V), joining (J), and constant (C) regions that rearrange in cells of the T lineage (*3*). In contrast to the TCRα and TCRβ genes, there appears to be a much smaller number of Vγ elements (see below) (*4*), implying that TCRγ can make only a limited contribution to the diversity of any receptor involving this chain. A clue to the possible function of the TCRγ product came from the finding that γ messenger RNA (mRNA) is expressed at high levels in early murine fetal thymocytes and analogous Lyt2$^-$, L3T4$^-$ cells from adult thymus (*5*). Since these populations contain precursors to mature T cells (*6*), the γ protein has been postulated to play a role in T cell development. In order to study TCRγ expression at the protein level, we have raised an antiserum to a synthetic peptide corresponding to the seven carboxyl-terminal amino acids of the murine γ chain; the peptide sequence was deduced from the complementary DNA (cDNA) sequence (*3*). Using this antiserum and a monoclonal hamster antibody to the murine T3 complex (termed 145-2C11), we show here that a subset of adult Lyt2$^-$, L3T4$^-$ thymocytes express γ chain on their surface in association with the T3 complex. A similar molecule has been identified in certain T cell populations of human peripheral blood (*7*). The murine γ chain is disulfide-bonded to a second 45-kD protein that is neither the α nor the β chain. All of the detectable γ chain on the surface of these cells is 35 kD and reduces to the predicted core size of 32 kD after treatment with endoglycosidase. This analysis demonstrates that there is no expression at the protein level of the $V_{\gamma 1.2}$-$C\gamma_2$ combination that is the major γ mRNA expressed outside the thymus. These T3$^+$, Lyt2$^-$, L3T4$^-$ cells may therefore represent a distinct lineage of cells within the thymus.

The murine γ gene family has been extensively characterized at the DNA level (*4, 8–10*). A schematic diagram of the genomic structure and probable rearrangement patterns is shown in Fig. 1. There are four known J-C elements ($J_1$-$C_1$, $J_2$-$C_2$, $J_3$-$C_3$, and $J_4$-$C_4$) and four known V region families ($V_1$, $V_2$, $V_3$, and $V_4$); each has one member, except for $V_1$, which has three members ($V_{1.1}$, $V_{1.2}$, and $V_{1.3}$). Furthermore, $V_2$, $V_3$, and $V_4$ each rearrange to $J_1$-$C_1$; $V_{1.2}$ rearranges to $J_2$-$C_2$; $V_{1.1}$ rearranges


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