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Immunology

Immunologic abnormalities of mice bearing the *gld* mutation suggest a common pathway for murine nonmalignant lymphoproliferative disorders with autoimmunity

(T-cell function/cell-surface antigens)

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ABSTRACT **Mice bearing the autosomal recessive mutation *gld* have been shown to develop massive lymphadenopathy, hypergammaglobulinemia, and autoantibodies and to die prematurely with interstitial pneumonitis. In this study, lymphocytes from C3H *gld* and C3H +/+ mice were examined for a variety of phenotypic and functional characteristics. Spleens and lymph nodes of mutant mice were expanded by an aberrant population of Ly-5(B220)$^+$ surface immunoglobulin negative cells that were Thy-1$^+$ Ly-1$^+$ or Thy-1$^-$ Ly-1$^+$. Cells from both tissues of mutant mice older than 8 wk were impaired in their ability to proliferate in response to allogeneic stimuli, and supernatants of cells stimulated with concanavalin A contained significantly reduced levels of interleukin 2. Cytotoxic T-lymphocyte responses of spleen and lymph node cells from C3H *gld* mice were normal at all ages tested. These results are strikingly similar to those obtained with C3H mice homozygous for the nonallelic autosomal recessive mutation *lpr*. We suggest that the similarities between the syndromes induced by these two mutations may reflect alterations in different enzymes that act in a common metabolic pathway of major importance to the differentiation and function of T cells.**

The mutant gene *gld* (*g*eneralized *l*ymphoproliferative *d*isease), has been shown to induce early-onset massive lymphoid hyperplasia, hypergammaglobulinemia, and production of antinuclear antibodies in C3H/HeJ (C3H) mice (1). This pattern of abnormalities resembles that induced by the *lpr* (lymphoproliferation) mutation (2, 3). However, *gld* and *lpr* are not allelic (1), and C3H mice bearing the *gld* mutation die with chronic interstitial pneumonitis (1), whereas C3H *lpr* mice do not develop this inflammatory pulmonary disease (unpublished observations).

We previously demonstrated that lymph nodes of *lpr* homozygous mice on different genetic backgrounds are expanded by a unique population of lymphocytes (4, 5). These cells express Ly-5(B220), a cell-surface antigen detected by the monoclonal antibody 6B2, that is expressed only on cells within the B-lymphocyte lineage of normal mice (6). However, surface immunoglobulin-negative (sIg$^-$) cells of MRL-*lpr* and C3H-*lpr* mice have no rearrangements of Ig heavy-chain genes, clearly demonstrating that they are not of B-cell origin (4). The Ly-5(B220)$^+$, sIg$^-$ cells are probably of T-cell origin in that (*i*) development of lymphadenopathy is abrogated by neonatal thymectomy (7, 8), and (*ii*) most of these cells are Thy-1$^+$ Ly-1$^+$ or Thy-1$^-$ Ly-1$^+$ (4, 5).

Supernatants of lymph node cells from *lpr/lpr* mice stimulated with concanavalin A (Con A) *in vitro* contain significantly less interleukin 2 (IL-2) than those of stimulated cells from +/+ mice (5, 9, 10) and proliferative responses to alloantigens in mixed lymphocyte reactions (MLR) are reduced (10, 11). In addition, cytotoxic T-lymphocyte (CTL) responses to alloantigens are depressed in some strains homozygous for *lpr* (5, 12) but are normal in others (5, 9–11).

In the current study, lymphocytes from C3H *gld* mice were examined for a variety of phenotypic and functional characteristics. The results showed that mice with this mutation had many immunologic abnormalities in common with C3H mice bearing the *lpr* mutation.

The observation that two non-allelic autosomal recessive mutations induce markedly similar syndromes in mice suggests that *gld* and *lpr* affect a common metabolic pathway of major importance in the differentiation and function of T cells in normal mice.

MATERIALS AND METHODS

Mice. Inbred and mutant strains C3H/HeJ *gld/gld* (C3H *gld*) and C3H/HeJ +/+ (C3H +/+) mice were bred and maintained in the authors' research colony or were obtained from The Jackson Laboratory. BALB/cAnN mice were obtained from the colonies of the National Institutes of Health.

Antibodies. Fluorescein isothiocyanate (FITC)-labeled antibodies to Thy-1.2, Ly-1, and Ly-2 and biotin-labeled anti-Ly-1 were purchased from Becton Dickinson, Sunnyvale, CA. FITC-labeled and unlabeled monoclonal 6B2 antibodies to Ly-5(B220) were generous gifts from Robert Coffman (DNAX Research Institute, Palo Alto, CA) (6). Biotin-labeled 6B2 was prepared in our laboratory. FITC-labeled anti-ThB was prepared with purified antibodies obtained from cultures of the 53-9.2 cell line (13), a generous gift of Jeffrey Ledbetter (Stanford University, Stanford, CA). FITC-labeled goat anti-mouse Fab and sulforhodamine 101-labeled avidin (TR-avidin) were gifts of B. J. Fowlkes (Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Unlabeled monoclonal antibody to Ly-5 was prepared from supernatants of the 30 F-11 cell line obtained from J. Ledbetter.

Flow Microfluorometry (FMF). Single cell suspensions from spleen and peripheral lymph nodes were prepared and stained for FMF analyses as described (14). FMF was performed on a fluorescence-activated cell sorter (FACS II, Becton Dickinson) with argon and krypton lasers, using established techniques (6, 14). One-color analyses were performed on 4×10^4 viable lymphoid cells, as gated by both narrow forward-angle light scatter and uptake of propidium iodide. Two-color FMF assays were performed on 10^5

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Abbreviations: sIg, surface immunoglobulin; IL-2, interleukin 2; MLR, mixed lymphocyte reaction; CTL, cytotoxic T lymphocytes; FITC, fluorescein isothiocyanate; FMF, flow microfluorometry.

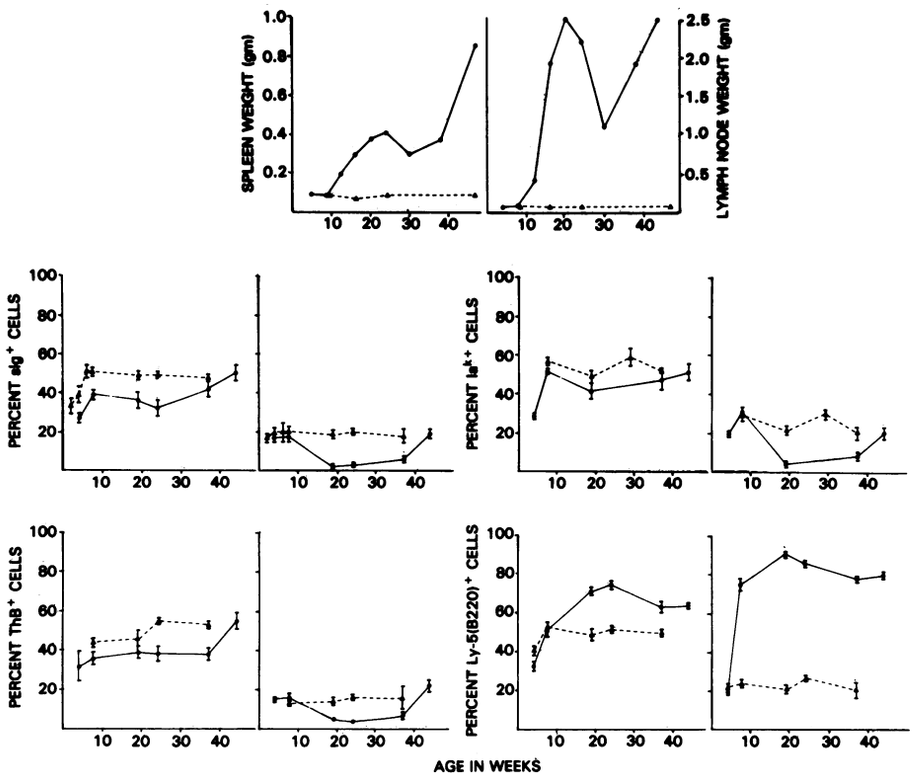

FIG. 1. In each of the paired panels in Figs. 1 and 2, values for spleen cells are given on the left and values for lymph node cells are given on the right. Solid lines, data for C3H *gld* mice; dashed lines, data for C3H +/+ mice. In lower four pairs of panels, values at each time point represent geometric mean ± 1 SEM (solid bars) for 3–8 individual mice tested by FMF with antibodies to the cell-surface marker given on the *y* axis. Upper pair of panels presents published data (1) reflecting the triphasic nature of *gld*-induced splenomegaly and lymphadenopathy.

lymphoid cells gated by light scatter alone. Two-color staining data are presented as contour plots on a 64 × 64 matrix.

IL-2 Production. Supernatants containing IL-2 were generated from cells stimulated with Con A (2 μg/ml) using the methods of Stader *et al.* (15) as modified in this laboratory (5). IL-2 activity was quantitated using the HT2 T-cell line as indicator cells and units of IL-2 activity were determined by regression analyses of test samples in comparison to a standard supernatant from BALB/c spleen cells stimulated with 2 μg of Con A and were given the arbitrary value of 100 units/ml (5).

Generation of CTL. CTL were generated *in vitro* and their activity was quantitated in a ^{51}Cr-release assay using Con A-activated spleen cell targets as described (16). The activity of CTL effector cells is expressed as cytotoxic units per culture. The derivation of these units has been described (16).

MLR. Proliferative responses of spleen and lymph node cells to irradiated (2000 R; 1 R = 2.58 × 10^{-4} colomb/kg) BALB/c spleen stimulators were determined by [^{3}H]thymidine incorporation after 4 days *in vitro* as described (17).

RESULTS

Ontogeny of Cell-Surface Markers in Spleen and Lymph Node. It was shown previously that the lymphoproliferative disease of C3H *gld* mice consists of three phases (1) (Fig. 1 *Upper*). The first phase occurs between 8 and 20 wk of age, and it is marked by significant increases in the weights of both spleen and pooled peripheral, retroperitoneal, and mesenteric lymph nodes. Between 20 and 30 wk, there is a regression in the sizes of both these lymphoid compartments. This stage is followed by a final phase of secondary lymphoproliferation for both spleen and lymph node.

The proportions of cells in spleen and lymph node bearing various cell-surface markers were determined by FMF for mice in the different phases of *gld*-induced disease and for age-matched controls. Studies of markers expressed exclusively [sIg, Ly-5(B220), ThB] or predominantly (Ia^k) by normal cells in the B-lymphocyte lineage are shown in Fig. 1. It should be noted first that, at any time point, the estimates of B-cell frequency obtained with three independent markers for B cells (sIg, ThB, or Ia) were in close agreement. These results are in keeping with the view that essentially all B cells are sIg^+, Ia^+, and ThB^+. During the initial proliferative and regressive phases of *gld*-induced lymphoproliferation, the frequencies of cells bearing these three markers were slightly decreased in spleen and were markedly decreased in lymph node as compared to controls. In both spleen and lymph node, the secondary proliferative phase of the disease was associated with a rebound in the frequencies of "classic" B cells to normal levels (Fig. 1).

For control +/+ mice at all ages tested, the frequencies of Ly-5(B220)$^+$ cells in spleen and lymph node were, as expected, in close agreement with the frequencies of sIg^+, Ia^+, and ThB^+ cells. However, in spleens from C3H *gld* mice after 8 wk and in lymph nodes after 4 wk, the frequencies of Ly-5(B220)$^+$ cells were significantly higher than the frequencies of classic B cells. The frequencies of Ly-5(B220)$^+$ cells were relatively constant through all three phases of the disease, although they tended to peak with the initial proliferative phase. These results demonstrate that, as for all strains bearing the *lpr* mutation (4, 5), spleens and lymph nodes of C3H *gld* mice were expanded with an unusual population of cells that expressed Ly-5(B220) but were sIg^-.

Immunoprecipitation studies have shown that the proteins recognized by anti-Ly-5(B220) monoclonal antibodies on the surfaces of normal B lymphocytes and sIg^- cells from *lpr* homozygous mice are of similar size (220,000 Da), suggesting that the same antigen is expressed on both cell types

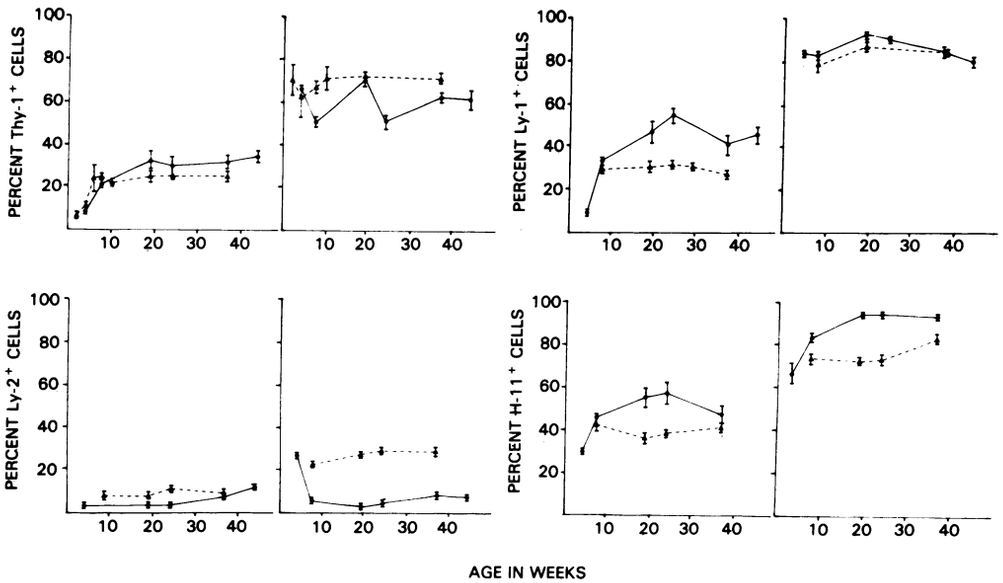

FIG. 2. See Fig. 1 legend. Values at each time point represent geometric mean ± 1 SEM (solid bars) for 3–8 individual C3H *gld* (solid lines) or C3H +/+ mice (dashed lines) tested by FMF with antibodies to the cell-surface marker given on the *y* axis.

(refs. 18 and 19; R. L. Coffman, personal communication). To confirm that the anti-Ly-5(B220) antibody used in the present study was also reactive with its nominal specificity, rather than a cross-reactive determinant of different molecular size, purified sIg^- and sIg^+ cells from C3H *gld* lymph nodes were surface-labeled with ^{125}I, and their lysates were precipitated with monoclonal antibodies to Ly-5 or Ly-5(B220). The results demonstrated that with either antibody, the major protein precipitated from the surface of sIg^+ and sIg^- lymph node cells was ≈220,000 Da (data not shown). These results indicate that, as for sIg^- cells from *lpr* homozygous mice, the predominant specificity recognized by anti-Ly-5(B220) antibodies on the surface of sIg^- cells from C3H *gld* mice is 220,000 Da.

Spleen and lymph node cells from C3H +/+ and C3H *gld* mice were also evaluated for the proportions of cells expressing cell-surface markers almost exclusively restricted to T cells (Thy-1, Ly-2) or expressed predominantly by T cells (Ly-1, H-11). The results of these studies (Fig. 2) showed that, although spleen cells from normal and mutant mice did not differ significantly in their content of $Thy\text{-}1^+$ cells, the frequencies of $Thy\text{-}1^+$ cells were always slightly higher in C3H *gld* mice older than 8 wk. By comparison, the frequencies of $Thy\text{-}1^+$ cells in lymph nodes of mutant mice were generally lower than in normal mice, but they tended to increase in relation to the two proliferative phases of the disease. As noted previously for C3H *lpr* cells (4, 5), the intensity of Thy-1 staining on C3H *gld* cells was significantly decreased as compared to normal (data not shown). $Ly\text{-}2^+$ cells in lymph node of C3H *gld* mice were significantly decreased in comparison to lymph nodes of normal mice at all ages after 4 wk; lesser reductions were also observed for $Ly\text{-}2^+$ cells in spleen up to 37 wk. The frequencies of $Ly\text{-}1^+$ spleen cells in C3H *gld* mice were increased above normal at all age points after 8 wk, but they remained near normal levels in lymph nodes. $Ly\text{-}1^+$ cells in normal and C3H *gld* lymph nodes stained with equal intensity (data not shown). These results differed from studies of $Ly\text{-}1^+$ cells in C3H *lpr* lymph nodes that showed them to stain with significantly lower intensity than normal (4, 5). Finally, the proportions of $H\text{-}11^+$ cells were increased over normal in C3H *gld* spleen and lymph node at all ages after 8 wk.

These findings demonstrated that during the various phases of *gld*-induced lymphoproliferative disease, the proportions of cells bearing the T-cell-related antigens Thy-1, Ly-1, and H-11 were present in near normal or slightly increased levels, but that the proportions of $Ly\text{-}2^+$ cells, particularly in lymph nodes, were decreased at most ages. Strikingly similar results were previously documented for mice homozygous for the *lpr* mutation (4, 5). Taken together, the data in Figs. 1 and 2 suggest that the major population of cells in lymph nodes of *gld* homozygous mice older than 4 wk was $Thy\text{-}1^+$, $Ly\text{-}1^+$, and $Ly\text{-}5(B220)^+$, and that these cells were present at a lower frequency among spleen cells.

Two-Color FMF Analyses of *gld* Lymph Node Cells. Lymph node cells from C3H *gld* and C3H +/+ mice 20 wk old were examined by two-color FMF assays for the expression of Thy-1, Ly-1, sIg, and Ly-5(B220). Two-color contour plots obtained with C3H +/+ and C3H *gld* lymph node cells stained with pairs of these reagents are shown in Fig. 3. Analyses of the patterns of cellular reactivity with both reagents are given in Table 1.

For cells from +/+ and *gld/gld* mice stained with anti-Thy-1 and anti-Ly-1 (Fig. 3 *A* and *B*), it was found that essentially all (>95%) $Thy\text{-}1^+$ cells were $Ly\text{-}1^+$ and that for +/+ lymph nodes, most (87%) of the $Ly\text{-}1^+$ cells were $Thy\text{-}1^+$. However, among C3H *gld* lymph node cells, 28% of the $Ly\text{-}1^+$ cells were $Thy\text{-}1^-$. Lymph node cells of C3H *lpr* mice also contain an increased population of $Thy\text{-}1^-$ $Ly\text{-}1^+$ cells (5).

Analyses of cells from C3H *gld* and controls stained with antibodies to sIg and Ly-5(B220) (Fig. 3 *C* and *D*) showed that essentially all sIg^+ cells were $Ly\text{-}5(B220)^+$ and that in controls, few of the $Ly\text{-}5(B220)^+$ cells (<1% of all cells) were sIg^-. However, in C3H *gld*, 96% of $Ly\text{-}5(B220)^+$ cells were sIg^-; $Ly\text{-}5(B220)^+$ sIg^- cells represented 89% of the total lymph node cell population in these mice (Table 1). Lymph nodes from C3H *lpr* mice also have an expanded population of $Ly\text{-}5(B220)^+$ sIg^- cells (4, 5).

Fig. 3 *E* and *F* shows the contours obtained with cells stained with anti-Thy-1 and anti-Ly-5(B220). As expected for normal cells, the lymph node subpopulations defined by these two reagents were almost entirely exclusive; only 1% of the cells were $Thy\text{-}1^+$ $Ly\text{-}5(B220)^+$. By comparison, 62% of all C3H *gld* lymph node cells were $Thy\text{-}1^+$ $Ly\text{-}5(B220)^+$ (Fig. 3*F*); 91% of all $Thy\text{-}1^+$ cells had this phenotype (Table 1). Similar studies of C3H *lpr* lymph nodes also showed that the majority of $Thy\text{-}1^+$ cells were $Ly\text{-}5(B220)^+$ (4, 5).

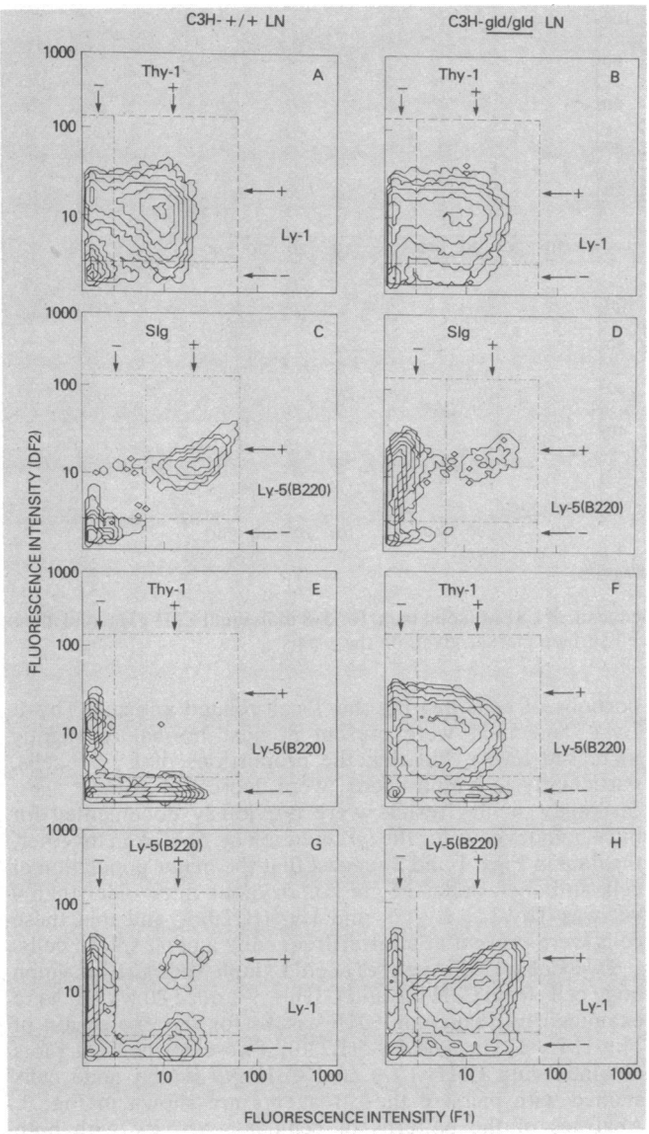

FIG. 3. Two-color FMF analyses of 20-wk-old C3H +/+ and C3H *gld* lymph node cells stained with antibodies labeled with FITC (F1) or biotin reacted with TR-avidin (DF2). Gates used to distinguish antigen-positive (+) from antigen-negative cells (−) are presented as dashed lines and were determined from single parameter fluorescence profiles.

Finally, comparisons were made between normal and C3H *gld* lymph node cells stained with anti-Ly-5(B220) and anti-Ly-1 (Fig. 3 *G* and *H*). In normal lymph nodes, only 5% of Ly-1$^+$ cells were Ly-5(B220)$^+$ and 38% of Ly-5(B220)$^+$ cells were Ly-1$^+$. In contrast, 87% of C3H *gld* lymph node cells were Ly-1$^+$ Ly-5(B220)$^+$; 94% of the Ly-5(B220)$^+$ cells and 93% of Ly-1$^+$ cells were of this phenotype (Table 1). Similar results were reported for two-color analyses of C3H *lpr* lymph node cells (5).

To summarize the two-color analyses, it was found that lymph node cells obtained from C3H *gld* mice at the peak of the initial lymphoproliferative phase of their disease (20 wk) included a major cell population with phenotypes rarely, if ever, expressed by normal lymph node cells. These aberrant cells were Ly-5(B220)$^+$ Ly-1$^+$ Thy-1$^+$ sIg$^-$ or Ly-5(B220)$^+$ Ly-1$^+$ Thy-1$^-$ sIg$^-$.

Functional Characteristics of C3H *gld* Lymphocytes. Spleen and lymph node cells from normal and mutant mice 7 to 44 wk old were tested for proliferative responses (MLR) and generation of CTL in response to stimulation with irradiated BALB/c spleen cells and production of IL-2 after stimulation with Con A (Table 2). In all three systems, the responses of 7- to 8-wk-old C3H *gld* mice did not differ significantly from those of age-matched +/+ mice except for the decreased capacity of spleen cells to produce IL-2. For mice older than 8 wk, the MLR responses of both spleen and lymph node cells of C3H *gld* mice were significantly lower than those of +/+ mice. In spite of their impaired ability to proliferate in response to allogeneic stimulation, spleen and lymph node cells from C3H *gld* mice of all ages gave allogeneic CTL responses equal to or, in some cases, significantly greater than those of age-matched controls (Table 2). Levels of IL-2 in supernatants of spleen and lymph node cells stimulated with Con A were significantly lower for C3H *gld* as compared to control mice at all ages except for lymph nodes from 7- to 8-wk-old mice.

Table 1. Two-color FMF analyses of lymph node cells from C3H +/+ and C3H *gld/gld* mice

FITC-labeled antibody to	Mice	Antibody reactivity	TR-avidin-biotin antibody to Ly-1 +	Ly-1 −	Ly-5(B220) +	Ly-5(B220) −
Thy-1.2	+/+	+	78	2	1	73
		−	12	9	15	11
	gld/gld	+	67	3	62	6
		−	26	4	30	2
sIg	+/+	+			13	<1
		−			1	85
	gld/gld	+			4	<1
		−			89	8
Ly-5(B220)	+/+	+	5	8		
		−	84	3		
	gld/gld	+	87	5		
		−	6	2		

See Fig. 3 legend for details of cell staining. Numbers indicate percent of cells reactive (+) or nonreactive (−) with each combination of reagents used in Fig. 3 and included in the boxed areas on each panel.

DISCUSSION

The results of this study and earlier investigations demonstrate that the phenotypic and functional abnormalities exhibited by C3H mice homozygous for the *gld* mutation were strikingly similar to those of lymphocytes from C3H mice homozygous for the nonallelic mutation *lpr* (1, 5, 9–12, 20). First, lymph nodes and spleens from mice with both mutations were expanded by populations of cells that are sIg$^-$ Ly-5(B220)$^+$ and either Thy-1$^+$ Ly-1$^+$ or Thy-1$^-$ Ly-1$^+$ (refs. 4 and 5; Figs. 1–3). Although cells with these phenotypes have not been detected among lymph node and spleen cells of normal C3H mice, it is still not proven that they are T cells that aberrantly express Ly-5(B220) during differentiation rather than being a markedly expanded subset of T cells that are present in normal tissues at frequencies below our limits of detection.

Second, cells from lymph node and spleen of both mutant strains were greatly impaired in their ability to proliferate in response to allogeneic stimuli (refs. 10 and 11; Table 2) and in the levels of IL-2 found in culture supernatants after stimulation with Con A (refs. 5, 9–11; Table 2). Third, allogeneic CTL responses of mice with either mutation remained near normal throughout their lives (ref. 5; Table 2; unpublished observations). Finally, C3H mice homozygous for either mutation have hypergammaglobulinemia and produce anti-nuclear antibodies (1, 20, 21).

Table 2. Functional assays of spleen and lymph node cells from C3H +/+ and C3H *gld/gld* mice

Assay	Cell source	Age, wk 7–8	20	28	36	44
MLR	+/+ spleen	33 ± 7	49 ± 10	46 ± 7	75 ± 3	38 ± 3
	gld/gld spleen	18 ± 7	9 ± 1*	8 ± 2*	4 ± 1*	7 ± 2*
	+/+ LN	71 ± 10	110 ± 22	170 ± 31	127 ± 9	163 ± 2
	gld/gld LN	61 ± 13	7 ± 2*	31 ± 12*	15 ± 4*	17 ± 5*
CTL	+/+ spleen	591 ± 21	355 ± 39	524 ± 61	346 ± 108	325 ± 20
	gld/gld spleen	625 ± 80	987 ± 29*	711 ± 114	858 ± 319	1759 ± 549*
	+/+ LN	399 ± 47	245 ± 31	294 ± 54	146 ± 16	167 ± 22
	gld/gld LN	510 ± 46	145 ± 65	206 ± 95	72 ± 28	216 ± 85
IL-2	+/+ spleen	24 ± 1	70 ± 17	84 ± 2	69 ± 3	62 ± 2
	gld/gld spleen	15 ± 3*	20 ± 5*	12 ± 2*	8 ± 2*	9 ± 4*
	+/+ LN	5 ± 1	8 ± 1	12 ± 6	9 ± 2	18 ± 1
	gld/gld LN	4 ± 1	<1*	2 ± 1*	1 ± 1*	5 ± 2*

Numbers indicate mean ± 1 SEM for 3–8 mice tested at each time point. MLR values are cpm × 10^{-3}; CTL values are cytotoxic units per culture; IL-2 values are IL-2 units/ml. LN, lymph node.
*Significantly different from C3H +/+ mice at $P < 0.05$.

In spite of these marked similarities between C3H mice homozygous for these two mutations, there are significant differences in the patterns of lymphoproliferation and histopathology exhibited by these animals. First, the lymphoproliferation exhibited by C3H *lpr* mice persists at high levels after 10 wk of age, whereas that of C3H *gld* mice is triphasic (ref. 1; Fig. 1). Second, C3H *gld* mice die with chronic interstitial pneumonitis (1), whereas C3H *lpr* mice do not.

When two or more mutations produce a similar clinical syndrome, genetic heterogeneity is said to exist. Genetic heterogeneity may result from different mutations at a single genetic locus (allelic heterogeneity) or from mutations at different genetic loci (nonallelic heterogeneity). The striking similarities in the phenotypic and functional abnormalities of lymphocytes from mice homozygous for the unlinked autosomal recessive mutations *lpr* and *gld* appear to represent an example of nonallelic heterogeneity.

The basic biochemical lesions underlying autosomal recessive disorders usually involve enzymatic proteins. In diseases with nonallelic heterogeneity resulting from autosomal recessive mutations, the unlinked defects have often been found to affect different enzymes that catalyze specific reactions in a single metabolic pathway (e.g., galactosemia due to galactokinase or galactose-1-phosphate uridyl transferase deficiency or immunodeficiency due to adenosine deaminase or purine nucleoside phosphorylase deficiency) (22, 23). The results obtained from studies of the *lpr* and *gld* mutations can therefore be interpreted to suggest that these mutations affect different enzymes in a common metabolic pathway of major importance to the differentiation and function of T cells. Alternatively, these abnormalities could be related to defects in completely unrelated genes. In either case, further studies of these mice could lead to the understanding of lymphoproliferative syndromes with autoimmunity as distinct metabolic abnormalities.

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