A NEW MUTATION, gld, THAT PRODUCES LYMPHOPROLIFERATION AND AUTOIMMUNITY IN C3H/HeJ MICE

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The reports by Bielschowsky et al. in 1959 (1) on the natural history of autoimmune disease in NZB mice and by Helyer and Howie in 1963 (2) on the autoimmune disease with systemic lupus erythematosus-like glomerulonephritis in the (NZB × NZW)F₁ hybrid provided the foundation for extensive studies on genetic, immunologic, and virologic factors in the pathogenesis of systemic murine lupus (3, 4). Although subsequent studies have documented multiple functional and phenotypic abnormalities of NZB T and B lymphocytes (5–8), only a few independently segregating loci have been identified that influence the expression of each of these abnormalities (5, 9–14) and only one gene has been assigned to a chromosomal location (15).

The opportunity to study the effects of single genes on autoimmune disease has been provided by the development of mouse strains bearing the unmapped autosomal recessive mutation lymphoproliferative (Ipr) (16–17) and the Y chromosome-linked autoimmune acceleration factor (Yaa) of strain BXSB/Mp (17–18). Mice homozygous for the lpr mutation or BXSB male mice develop massive lymphadenopathy, immune complex glomerulonephritis, hypergammaglobulinemia, and autoantibodies (16–18) and have been the models of choice for studies of autoimmunity in many laboratories (9, 11, 12, 19, 20).

A new spontaneous autosomal recessive mutation that occurred in the inbred mouse strain C3H/HeJ determines the development of an early onset lymphoproliferative disorder with autoimmunity (21). This new mutation has been named generalized lymphoproliferative disease, gene symbol gld. Data on its natural history and basic immunopathology are reported here. This new single-gene model for autoimmune disease may help identify critical immunoregulatory pathways and common pathogenetic mechanisms of generalized autoimmune disease in mouse and man.

Materials and Methods

Mice. Inbred and mutant strains C3H/HeJ-gld/gld, C3H/HeJ-+/+, MRL/Mp-lpr/lpr, C3H/HeJ-lpr/lpr, BXSB/Mp, NZB/BINJ, C56BL/6J, and SJL/J were bred and maintained in the authors' research colony. All mice were fed a diet of Old Guilford 96 pelleted feed (Emory Morse Co., Guilford, CT) and received chlorinated water ad libitum.

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**Genetics.** The specific crosses used to localize the gld mutation to chromosome 1 are given in Results. Segregating loci included the skeletal mutation Lp (loop tail) and two isozyme loci, Idh-1 (isocitrate dehydrogenase-1) and Pep-3 (peptidase-3, formerly called dipeptidase-3, Dip-3). The tissue used to determine Idh-1 and Pep-3 was kidney. Single kidneys were homogenized in 0.5 ml distilled water and the lysate centrifuged at 27,700 g for 30 min. The resulting supernatants were applied directly to Titan III cellulose acetate plates (Helena Laboratories, Beaumont, TX) and electrophoresis was conducted, cathode to anode, at 200 V for 30 min in a pH 8.5 Tris-glycine buffer. Staining for IDH-1 was accomplished essentially as described by Harris and Hopkinson (22). Staining for PEP-3 was accomplished as described in Eppig and Eicher (23).

**Lifespan Study.** All mice were examined weekly by palpation beginning at 6 wk of age. Mice were autopsied when moribund. Prior to autopsy, mice were exsanguinated via the retroorbital sinus. Erythrocyte and leukocyte counts were determined using a Coulter cell counter (Coulter Electronics, Inc., Hialeah, FL). Differential leukocyte counts were based on examination of 200 cells per Giemsa-stained blood smear. Packed erythrocyte hematocrits (HCT) were determined. Serum was processed and stored at −60°C for later analysis.

**Cross-Sectional Study.** Groups of five or six male gld/gld mice were killed at 4-wk intervals spanning 4–24 wk of age and at 30, 38, and 47 wk of age. Similar numbers of female gld/gld and of male and female congeneic +/+ controls were examined at 8, 16, 24, and 47 wk of age. Peripheral blood counts, including differential leukocyte analyses, were determined. Serum was processed as above and assayed for antinuclear antibodies (ANA), anti-double-stranded DNA (dsDNA), thymocyte-binding autoantibody (TBA), serum proteins, and immunoglobulin. Representative lymph nodes (mesenteric node complex, axillary, renal, and lumbar/caudal) were dissected, trimmed of extracapsular connective tissue, and weighed. The spleen and thymus were weighed. These organs as well as the liver, kidneys, mediastinal mass, and salivary glands, were fixed in Fekete’s formol-acetic-alcohol, embedded in paraffin, sectioned at 8 μm, and stained with hematoxylin and eosin (HE) for histological analysis. Sections of kidney were also stained with PAS/Schiff reagent.

**Lymphocyte Subset Analysis.** T and B cells were enumerated by fluorescence microscopy (24) and by flow microfluorometry (FMF) (25). In general, a pair of suspensions of mesenteric lymph node cells were prepared using Hank’s solution containing 10 mM Hepes, 5% fetal bovine serum, and 0.1% sodium azide, pH 7.2. One suspension was exposed to a 1:2 dilution of anti-Thy-1.2 antisera. Both conventional allogeneic and monoclonal anti-Thy-1.2 (cell culture supernatant from hybridoma HO-13-4.9 [26]) were used. The duplicate suspension was incubated with normal mouse serum (NMS). All suspensions were washed and exposed to a 1:10 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (FITC-GAMG) IgA + IgG + IgM, purchased from Cappel Laboratories, Cochranville, PA. The cells were again washed and then fixed in cold electron microscopy grade 2% formaldehyde solution. In microscopic analyses, 300 cells per suspension were scored as either dead, fluorescence positive, or negative. FMF analyses were conducted using an Ortho model 50H cytofluorograph (Ortho Diagnostic Systems, Inc., Westwood, MA). Data were registered in 512 channels from 50,000 cells using linear or logarithmic amplification.

**Serum Proteins and Immunoglobulin.** Total serum protein was determined by refractometry (27) using a temperature-compensated TS meter (American Optical Scientific Instruments, Buffalo, NY). Serum was applied to Titan III cellulose acetate plates and electrophoresed for 20 min at 180 V in a 0.05 M Tris-barbital/sodium barbital buffer (pH 8.8). Each plate was stained with Ponceau S, destained, and rendered transparent. Serum protein electrophoretic profiles were produced on a Helena Autoscan Densitom-
Quantitation of the immunoglobulin classes IgA, IgG1, IgG2a, IgG2b, and IgM was performed by agar-gel radial immunodiffusion (RID) using prepared 24-well plates from Meloy Laboratories, Inc., Springfield, VA. 4 μl of undiluted serum from 10 gld/gld and 8 control male and female mice, 20–24 wk of age, were applied in duplicate to RID plates. Precipitin rings were measured 48 h later. Ig levels were determined from concurrently prepared standard curves.

**Analysis of Autoantibodies.** Sera were tested for ANA by indirect immunofluorescence using acetone-fixed frozen sections of rat liver as the target substrate (28). The liver sections were overlaid with 75 μl of individual serum samples diluted to 1:10 (routinely) or doubling dilutions from 1:50 to 1:1,600 (for selected sera). After an incubation of 50 min at room temperature, the sections were washed with two changes of phosphate-buffered saline (pH 7.2) and overlaid with 100 μl of a 1:10 dilution of fluorescein-labeled IgG fraction of rabbit anti-mouse IgG (heavy and light chains) (FITC-RAMG). Before use, the FITC-labeled reagent was absorbed with mouse acetone liver powder and ultracentrifuged.

Selected sera were also evaluated for reactivity against native DNA using the kinetoplast of *Crithidia luciliae* (CL) (29). Prepared CL substrate slides were obtained from Meloy Laboratories, Inc. Indirect immunofluorescence techniques were used as described above. The intensity of fluorescence of liver nuclei or CL kinetoplast was scored as follows: (−), no specific fluorescence; (±), questionable; (+), weak but definite; (++), moderate; (+++), strong. Sera that scored ++ or greater were considered positive.

Analyses for TBA were performed by indirect immunofluorescence microscopy as described for lymphocyte subset analyses. Thymocytes obtained from 4–6-wk-old C3H/HeJ+/+ mice were incubated first with 1:2 dilutions of individual serum samples followed by 1:10 diluted FITC-GAMG.

**Statistical Analyses.** All measures of variance are reported as standard errors of the mean (except in Table 1 where the standard deviation is used). Tests of significance for differences between independent means were performed by Student's *t* test.

**Results**

**Genetics.** Linkage to *Pep-3* (peptidase-3) was first noted in a backcross involving (C57BL/6By × C3H/HeJ-gld)F1 mice mated to C3H/HeJ-gld mice. Out of a total of 196 mice analyzed, 170 inherited a parental combination of the *Pep-3* and *gld* alleles from their F1 parent (93 *Pep-3*+, 77 *Pep-3* gld) and 26 inherited a recombinant combination (18 *Pep-3* gld, 8 *Pep-3*+), indicating *gld* was located on chromosome 1 with 13.3 ± 9.4% recombination between it and *Pep-3*.

To determine the order of *gld* relative to other chromosome 1 genes, three other crosses were made. In cross 1, C57BL/6Ks females were mated to C3H/HeJ-gld males and the resulting (Idh-1/*Pep-3*+/Idh-1/*Pep-3* gld)F1 females were backcrossed to C3H/HeJ-gld males. In cross 2, a recombinant male (*Pep-3* gld/*Pep-3* gld) from cross 1 was mated to a *Lp*/+ female. Offspring that were *Lp*/+ and carried the *Pep-3* allele (ie., *Pep-3* gld+/*Pep-3* gld) were mated to C3H/HeJ-gld/gld mice and the offspring classified for the three segregating loci. In cross 3 several *Pep-3*+/*Lp*/+ individuals from cross 2 were mated to C3H/HeJ-gld/gld females and the offspring analyzed. The results of these three crosses are presented in Table 1. We conclude that *gld* is located on chromosome 1 between the genes *Pep-3* and *Lp*.

**Lifespan Analysis.** 24 male and 25 female C3H/HeJ-gld/gld and 25 female +/+ control mice were held for lifespan studies. Enlarged peripheral lymph nodes were readily palpable in *gld* homozygotes of both sexes by 13 wk of age. Enlarged abdominal lymph nodes and splenomegaly were evident shortly thereafter. An
### Table 1

**Analysis of Chromosomal Linkage of the gld Mutation**

**Cross 1**

| ldh-1 Pep-3 gld | Region of recombination | Total offspring |
|----------------|-------------------------|-----------------|
| a b gld        | None                    | 61              |
| b a +          |                         | 54              |
| a a +          | Idh-1-Pep-3             | 25              |
| b b gld        | Pep-3-gld               | 13              |
| b a gld        | Idh-1-Pep-3             | 10              |
| a a gld        | Pep-3-gld               | 6               |

Gene order: *Idh-1-31.0 ± 3.3-Pep-3-15.0 ± 3.5-gld*

**Cross 2**

| Pep-3 gld Lp  | Region of recombination | Total offspring |
|---------------|-------------------------|-----------------|
| a gld +      | None                    | 39              |
| b + Lp       |                         | 35              |
| a + Lp       | Pep-3-gld               | 9               |
| b gld +      |                         | 3               |
| a gld Lp     | gld-Lp                  | 10              |
| b + +        |                         | 4               |

Gene order: *Pep-3-12.0 ± 3.2-gld-14.0 ± 3.5-Lp*

**Cross 3**

| Pep-3 gld Lp  | Region of recombination | Total offspring |
|---------------|-------------------------|-----------------|
| a + Lp       | None                    | 43              |
| b gld +      |                         | 35              |
| a gld +      | Pep-3-gld               | 12              |
| b + Lp       |                         | 3               |
| a + gld-Lp   |                         | 2               |
| b gld Lp     |                         | 3               |

Gene order: *Pep-3-15.8 ± 3.6-gld-5.1 ± 2.2-Lp*

* Percent recombination ± SD.

† Data combined from heterozygous female and male parents.
episodes of marked regression of lymph node enlargement was observed during subsequent periodic inspections (documented in detail in a separate cross-sectional study described below). Both male and female gld/gld mice have a protracted clinical course and survive to a mean age of 396 ± 13 and 368 ± 18 d, respectively (not significantly different by t test analysis). The longevity of female +/+ control mice was nearly double (688 ± 35 d) that of female gld/gld mice (P < 0.001) (Fig. 1).

28 of the 49 gld homozygotes used in the lifespan study were autopsied when moribund. Seven of these had marked subcutaneous edema. At autopsy, all C3H-gld mice presented enlargement of all lymph nodes and splenomegaly. The liver and kidneys appeared normal. Anemia (HCT, <30%) or significant leukocytosis (>25 × 10⁹/mm³) were found in 40% of those autopsied. Differential blood counts were determined for 14 male gld/gld mice autopsied when moribund. The number of lymphocytes (18.1 × 10⁹/mm³) were nearly fourfold greater and the number of neutrophils (5.2 × 10⁹/mm³) was double that of baseline +/+ control values (for comparison, see Fig. 4a and b).

**Histopathology.** All lymph nodes were massively enlarged. Histological analysis revealed a loss of nodular architecture with a predominant proliferation of lymphocytes including an admixture of histiocytes and plasma cells (Fig. 2a–c). Fibrosis and multinucleated giant cells also were frequently observed. This range of pleomorphism and chronic inflammatory pattern was not observed at the peak of primary lymphoproliferation (i.e., 20 wk old).

To determine whether these lymphoid lesions were neoplastic, eight groups containing six young adult C3H+ mice were inoculated subcutaneously or intraperitoneally with 20–40 mg boluses of lymph node tissue from individual 5-mo-old C3H-gld donors. These recipients were examined biweekly for evidence of growth of the transplanted (subcutaneous or intraperitoneal) lymphoid tissue. No palpable growths were detected. 45 wk after transplant, all recipients were killed and examined for evidence of transplant growth. None of 48 recipients had gross evidence of lymphoma, either spontaneous or transplanted. Although

![Figure 1](image.png)

**Figure 1.** Longevity of C3H/HeJ gld and coisogenic C3H/HeJ+ female mice. Mutant gld mice live only one-half as long as controls (P < 0.001).
it does not appear that the gld/gld lymphoid lesion is neoplastic, the possibility of its being prelymphomatous has not been ruled out. Infiltration of lymphoid cells into the perivascular tissue of the kidney, liver, salivary glands, pancreas, and other nonlymphoid organs was infrequent or absent. Minor accumulations of lymphocytes in peribronchiolar sites was seen rarely. The erythroid and myeloid components of femoral bone marrow appeared normal and there was no obvious infiltration by lymphoid cells. The increase in spleen size can be attributed primarily to the expansion of the white pulp.

Virtually all C3H-gld mice autopsied had an extensive inflammatory disease of the lung, resembling interstitial pneumonitis (Fig. 2d-f). Typically, large numbers of well-differentiated lymphocytes and macrophages accumulated in the alveolar septa, first in the periphery and later centrally in the lobe. The alveoli were not collapsed. Neither alveolitis nor bronchiolitis was found. In a few cases, focal granulomatous reaction with giant cells was observed. Extensive necrosis was rare. Accumulations of eosinophils have not been observed. Analyses of the lungs of young adult (8–30-wk) gld/gld mice suggested the following general pattern of development of pulmonary pathology: Minor focal hemorrhages and congestion of alveolar capillaries was followed by a moderate infiltration of the interstitial tissue with polymorphonuclear leukocytes (PMN). Later, macrophages accumulate in the alveolar septa, replacing PMN. Finally, large numbers of well-differentiated lymphocytes intermix with the macrophages. Perivascular infiltration of lymphocytes was not associated with this inflammatory process.

Histologic examination of the kidneys of moribund C3H-gld mice revealed a highly variable degree of change to the glomeruli and tubules. A composite score indicating the overall degree of lupus-like renal disease was assigned to each of 28 cases examined. The criteria were: (−) negative, no obvious renal disease, (+) minor focal glomerular change, (++) moderate glomerular disease, (+++) extensive glomerular disease with or without tubular pathology. Among the histologic features considered were hypercellularity, necrosis, hyalinization, capillary thickening, presence of inflammatory (PMN) cells, capsular adhesions, glomerular dilation, tubular dilation, and casts. Both HE- and PAS/Schiff-stained sections were examined. Only 4 of 28 (14%) of those kidneys from C3H-gld mice had moderate to extensive lupus-like disease. The majority, 15/28 (54%), had either none or only minor focal changes.

We examined cryostat sections of kidneys from eleven gld/gld mice between 8 and 41 wk old by direct immunofluorescence microscopy using polyclonal FITC-GAMG. Significant depositions of immune complexes were found in the glomeruli (primarily in the mesangium) of all C3H-gld mice 22 wk old or older. This deposition of immune complexes is not necessarily a prelude to significant renal disease later in life, since the immune complex nephritis characteristic of the BXSB, MRL-lpr, and NZB/W lupus models is not a common feature of the pathology of the C3H gld mutant mouse.

Vascular disease including necrotizing arteritis or polyarteritis was not observed in our examinations of gld/gld mice. Erythema and necrosis of the ears and nuchal dermatitis so common in MRL lpr mice is not observed in C3H-gld
FIGURE 2. Histopathology of C3H/HeJ-gld mice. Hematoxylin and eosin. (a–c) Lymph node. (a) 22-wk-old gld/gld male. Lymph node is greatly enlarged with diffuse proliferation of lymphoid cells with loss of normal architecture. Clusters of lymphoid cells are often observed (×27). (b) Higher magnification (×270) showing the predominance of mature lymphocytes and immunoblasts. (c) 63-wk-old gld/gld male (×270). The cellularity of the enlarged lymph nodes is pleomorphic, with macrophages, plasma cells, and foreign body giant cells (arrow) in evidence. Moderate fibrosis is often observed. Sinusoids may be engorged with blood. (d–f) Lung from 63-wk-old gld/gld male. (d) Low magnification (×27) showing extensive interstitial inflammatory disease. There is no bronchiolitis and alveolitis. (e) Higher magnification (×270). The earliest changes are capillary engorgement and infiltration of the interstitium with polymorphonuclear leukocytes. (f) More advanced lesions usually consist of extensive accumulations of macrophages (arrows) and well-differentiated lymphocytes (×270). PMN have been replaced. Focal hemorrhage as well as granulomatous lesions with foreign body giant cells (not shown) are common at this age.
mice.

**Chronology of Lymphoproliferation.** The weights of the mesenteric node complex, left and right axillary, left and right renal, and the lumbar/caudal lymph nodes from individual mice were added and presented as the "combined lymph node weight" (Fig. 3). By 8 wk of age, a significant increase in lymph node weight was found for both male (76 mg, \( P < 0.05 \)) and female (66 mg, \( P < 0.001 \)) gld homozygotes compared with normal male (53 mg) or female (45 mg) +/- controls. By 12 wk of age, the lymph nodes of male C3H-gld mice were ninefold heavier than those of controls. By 20 wk, they reached 2,530 mg, a 60-fold increase compared with +/- controls. A major regression of lymph node mass (originally observed during periodic palpation of mice held for lifespan studies) was evident from this cross-sectional analysis. By 30 wk of age, male C3H-gld mice had a combined lymph node weight of 1,050 mg, a significant reduction (\( P < 0.005 \)) of 1,500 mg compared with the weight 10 wk earlier (the peak of primary lymphoproliferation). A significant rebound was evident by 38 wk of age, with lymph node weights increasing by 820 mg from the 30-wk-old low (\( P < 0.025 \)). The increase in lymph node mass reached nearly 3,000 mg (96-fold increase over age-matched male controls) by 47 wk. There was no significant difference in the lymph node weight of female as compared with male gld/gld mice at any age evaluated (8, 16, 24, and 47 wk).

Significant splenic enlargement was evident as early as 12 wk of age in male (\( P < 0.005 \)) gld/gld mice (Fig. 3). At 16 wk of age, gld/gld females and males had 2.9- and 4.1-fold increases over their respective controls. By 47 wk, the spleens of gld males were 7.5-fold heavier than controls. The most accelerated phase of splenic enlargement occurred between 38 and 47 wk of age. Unlike the lymph node mass, the spleen did not undergo a pronounced episode of enlargement-regression-rebound (Fig. 3).

There was no significant difference at any age tested between the thymus weights of gld/gld mutant mice and of age- and sex-matched +/- controls. There

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**FIGURE 3.** Changes in lymph node (— — —) and spleen (— — —) weight with age in C3H-gld (○) and C3H+ (○) male mice. Lymph node weight represents the summation of mesenteric, axillary, renal, and the lumbar/caudal lymph nodes. Five or six mice were used for each age point. Several stages of the gld lymphoproliferative process can be identified: preproliferative (<8 wk), primary proliferative (12–20 wk), regressive (20–30 wk), and a secondary proliferative phase (30–47 wk).
was no significant difference at any age in body weight between genotypes of either sex.

**Hematology.** Hematologic evaluations were conducted on C3H-gld male mice at 4-wk intervals from 4- to 24 wk of age and also at 30, 38, and 47 wk of age. The total leukocyte count of male gld/gld mice rose from $7.1 \times 10^3$/$\text{mm}^3$ at 4 wk to a maximum of $25.4 \times 10^3$/$\text{mm}^3$ at 24 wk of age. At 47 wk, this leukocytosis was reduced to $15.2 \times 10^3$/$\text{mm}^3$. The total leukocyte counts of 20 male +/- mice ranging in age from 8 to 47 wk was $8.0 \pm 0.4 \times 10^3$/$\text{mm}^3$.

The number of circulating lymphocytes rose dramatically, reaching $19.1 \times 10^3$/$\text{mm}^3$ at 24 wk of age (Fig. 4a). The change with age in numbers of circulating lymphocytes corresponds closely with changes in lymph node weight. The regression in lymph node mass is paralleled by a significant reduction of circulating lymphocytes, from 19.1 to $9.9 \times 10^3$ cells/$\text{mm}^3$, between 24 and 30 wk of age ($P < 0.01$). Microscopically, the majority of lymphocytes were small well-differentiated cells. Male C3H-+ control mice had an average of $5.0 \pm 0.3$ × $10^3$/$\text{mm}^3$ lymphocytes at 12–47 wk of age. The number of circulating lymphocytes in male C3H_gld mice was significantly greater ($P < 0.005$) than in controls 16 wk of age and older. The number of neutrophils was significantly greater (two to threefold) in male gld/gld compared with +/- mice, from 8 to 38 wk old (Fig. 4b). Immature polymorphonuclear forms were uncommon.

At 47 wk the number of circulating erythrocytes had decreased to 88% of normal in both sexes. Packed cell volumes were similarly reduced to ~93% of normal in both sexes. This modest normocytic anemia was not observed during the peak of primary lymphoproliferation (20 wk).

**Lymphocyte Subsets.** 24 gld/gld males between 8 and 39 wk old were killed and mesenteric lymph nodes were removed for immunofluorescence analysis to determine the frequency and numbers of surface Ig (sIg)-positive (B cells), Thy-1-positive (T cells), and sIg- and Thy-1-negative (null) lymphoid cells. The mesenteric lymph nodes of 19 adult (8–39 wk old) +/- males were included in this study. At 18–24 wk of age, there was a significant increase above normal (+/+) in the absolute number of all three lymphoid subsets for this population of gld homozygotes. The frequency of B cells decreased from 31.3 to 14.3%; T cells increased from 61.5 to 67.9%; and null cells increased from 7.1 to 17.8%. The number of viable cells obtained from the mesenteric node complex of C3H-gld mice was $331 \times 10^6$ compared with $26 \times 10^6$ for C3H-+ controls. By extrapolation, absolute numbers of B, T, and null lymphocytes in gld/gld (compared with +/-) mice increased by 6-, 15-, and 33-fold, respectively (Table II).

FMF analysis of age-matched pairs of mutant and control lymphocyte suspensions confirmed the observations made by fluorescence microscopy. Typically, FMF produced histograms that confirmed the decreased frequency of B cells and the increase in frequency of T cells and null cells (Fig. 5a and b).

**Serum Proteins and Immunoglobulin.** The amount of total serum protein was similar for gld/gld and +/- mice. 18 male gld/gld mice between 18 and 24 wk old had $7.01 \pm 0.13$ g protein/dl of serum compared with $7.06 \pm 0.08$ g for 14 adult +/- male controls.

Electrophoretic analysis of serum revealed a prominent increase with age of gamma region proteins from gld homozygotes. The sera of 34 gld/gld and 10
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FIGURE 4. Peripheral leukocyte counts in C3H gld (---) and normal (----) male mice. Mean numbers of leukocytes ± SE from groups of five or six gld/gld mice (46 total). Because the numbers of each cell type remained constant in control mice (8-47 wk old), the data was pooled (n = 20). (a) Lymphocytes. The fluctuation in numbers of lymphocytes with age recapitulates the changes in lymph node mass with age (see Fig. 3). (b) Neutrophils. There was a significant increase in the number of peripheral blood neutrophils (two to threefold) in gld/gld mice compared with controls at all ages examined (8-38 wk).

TABLE II
Analysis of the Major Lymphocyte Subsets in C3H-gld Male Mice

| Genotype | Age (weeks) | Number of mice | Numbers of mesenteric lymph node cells (× 10⁶) |
|----------|-------------|----------------|-----------------------------------------------|
|          |             |                | All cells | B cells* | T cells† | Null cells$^\ddagger$ |
| +/+      | 8-39        | 19             | 25.2 ± 2.3† | 7.9 ± 0.8 | 15.5 ± 1.6 | 1.8 ± 0.2 |
| gld/gld  | 8-9         | 8              | 37.1 ± 5.0  | 9.2 ± 1.5 | 23.7 ± 3.4 | 4.2 ± 0.9 |
| gld/gld  | 18-24       | 9              | 330.8 ± 39.4 | 47.3 ± 4.8 | 224.6 ± 28.5 | 58.9 ± 12.2 |
| gld/gld  | 33-39       | 7              | 87.7 ± 16.2 | 16.2 ± 3.5 | 53.7 ± 8.8 | 17.8 ± 5.2 |

* slg-positive.
† Thy-1 alloantigen-positive.
$^\ddagger$ slg- and Thy-1-negative.
$^\ddagger$ All values are expressed as mean ± SE.

+/+ males were examined by cellulose acetate electrophoresis. The gamma region proteins of gld/gld mice rose from 2.3% at 4-8 wk of age to 8.8% at 20-24 wk and 17% at 38-47 wk. None of the sera of C3H+ mice (24-47 wk old) had gamma globulin in excess of 3% of total protein. Analysis of densitometric traces indicates that the sera of clinically affected gld/gld mice have either electrophoretically broad or restricted gammopathies (Fig. 6a-d). All of 13 sera
from 20-24-wk-old gld/gld mice had broad migration of gamma region proteins similar to that in Fig. 6b. At 30-47 wk of age, one-half (8/16) of the sera tested from C3H-gld mice had electrophoretically restricted gammapathy similar to that in Fig. 6c. The majority of these sera had elevations of "slow" rather than "fast" (cathodic migration) gamma proteins. A few sera had bimodal distribution, as seen in Fig. 6d. Increases in beta globulin were not remarkable. None of the sera from clinically affected gld/gld mice had significant alterations of alpha region globulin.

All classes of immunoglobulin measured by RID were markedly elevated in 20-24-wk-old C3H-gld mice compared with age-matched controls (Fig. 7). The mean serum Ig levels (mg/ml) for eight C3H+ mice were: IgA, 0.21 ± 0.01; IgG1, 0.67 ± 0.05; IgG2a, 1.25 ± 0.32; IgG2b, 0.19 ± 0.04; and IgM, 0.36 ± 0.02. All of 10 sera from C3H-gld mice had at least a threefold increase in serum IgM. The proportion of C3H-gld mice with at least fivefold elevations in serum IgG1, IgG2b, IgA, and IgG2a were 30, 60, 70, and 80%, respectively. 9 of 10 clinically affected gld homozygotes had major increases in more than one immunoglobulin isotype. There appeared to be a mutually exclusive relationship between elevations of the IgA and IgG1 isotypes. The sera with the five highest IgA levels had low level IgG1. The three highest IgG1 levels corresponded with low level IgA (data not shown).
FIGURE 6. Cellulose acetate electrophoresis of serum from normal +/+ (a) and selected cases of mutant gld/gld (b–d) mice. An increase in gamma (γ) region proteins (vertical hatching) is found in nearly all clinically affected gld mice. ALB, albumin; α, alpha globulin; β, beta globulin.

FIGURE 7. Quantitation of the major serum immunoglobulin classes by RID. In a number of cases, the level of serum Ig exceeded the working limits of the system (as defined by the supplier). Rather than extrapolate beyond these limits, those individual high values are shown above a horizontal dashed bar indicating the appropriate upper limit. All C3H-gld mice had marked elevations of two or more Ig classes.
Cryostat sections of rat liver were incubated with 1:10 diluted sera from mutant gld (---) and control (-----) mice followed by exposure to FITC-labeled RAMG. Sera from five or six males were examined at each age indicated. Additionally, sera from eight gld/gld and six control females, 24-wk-old, were evaluated.

Figure 9. Frequency of anti-dsDNA autoantibody positivity. C. luciliae kinetoplast fluorescence determined after incubation with 1:10 diluted serum followed by FITC-GAMG. (a) Number of positive sera/number of examined sera. (b) Includes sera from four female C3H-gld or four female C3H-+ mice. (c) +/+ refers to the wild-type allele at both the gld and lpr loci.

Antinuclear Autoantibody. Autoantibodies directed against nuclear antigens were detected in sera from C3H-gld mice. Sera from all of the male gld/gld and +/+ mice killed at monthly/bimonthy intervals were evaluated for ANA. By 8 wk of age, two of the five gld/gld sera (diluted 1:10) assayed were positive for ANA (Fig. 8). Beginning at 16 wk of age, all of 40 sera from C3H-gld mice were positive. None of the sera from C3H-+ mice from 8 to 47 wk were positive. Sera from eight 24-wk-old C3H-gld mice were twofold serially diluted from 1:50 to 1:1,600. Five of eight of these sera had positive ANA activity at dilutions of 1:400.

Anti-dsDNA Autoantibody. Using the kinetoplast of C. luciliae as a dsDNA target in immunofluorescence studies, we found that serum of C3H-gld mice had levels of anti-dsDNA autoantibody similar to or exceeding that of other murine
models known to have high levels of this antibody (Fig. 9). All of 19 sera (diluted 1:10) from 12-24-wk-old C3H-gld mice were positive for anti-dsDNA autoantibody. All eight sera from mice homozygous for the lpr mutation and ~65% of sera from strain BXSB and NZB male mice also were positive for anti-dsDNA. None of the sera from 19 congeneric normal C3H-+/+, 15 C57BL/6, or 6 SJL/J nonautoimmune mice had anti-dsDNA activity.

Thymocyte-binding Autoantibody. The sera of C3H-gld mice contain significant levels of autoantibody to cell surface determinants expressed on young adult thymocytes. Autoantibody was detected on >50% of target thymocytes after incubation with 1:2 diluted sera from 20 wk or older gld/gld mice. Sera from congeneric +/+ control (from 4 to 47 wk old) mice had an average of 7% positive target thymocytes in this immunofluorescence assay (Table III). Approximately 50% of thymocytes were positive after incubation with sera from 1-yr-old NZB mice. Sera from clinically affected MRL-lpr mice or young adult C57BL/6J mice had negligible levels of TBA.

Discussion

The autosomal recessive mutation gld (generalized lymphoproliferative disease) induces the development of massive, early onset lymphoid hyperplasia with hyperimmunoglobulinemia and production of autoantibodies to native DNA. These basic elements of the gld phenotype resemble those of the disease induced by another autosomal mutation, lpr (lymphoproliferation) (16, 17). In this paper, we demonstrate that lpr and gld are not allelic. Linkage studies place gld on

| Table III |
|-----------|

Thymocyte-binding Autoantibody in C3H-gld/gld Mice*

| Strain | Genotype | Sex | Age in weeks |
|--------|----------|-----|--------------|
|        |          |     | 4-8 | 12 | 16 | 20 | 24 | 30-38 | 47 |
| C3H    | gld/gld  | Male | ±1.9 | ±1.9 | ±5.9 | ±9.2 | ±7.2 | ±10.5 | ±3.1 |
|        |          | (6)  |     | (5) | (5) | (14) | (10) | (6) |
| C3H    | +/+1     | Male | ±2.2 | ±2.8 | ±1.2 | ±3.1 |     |     |     |
|        |          | (5)  |     | (5) | (9) | (5)  |     |     |     |
| NZB    | +/+      | Male |     |     |     |     |     |     |     |
|        |          | female |     |     |     |     |     |     |     |
|        |          |     | ±0.9 |     |     |     |     |     |     |
| MRL    | lpr/lpr  | Female |     |     |     |     |     |     |     |
| C57BL/6| +/+      | Male |     |     |     |     |     |     |     |
|        |          | female |     |     |     |     |     |     |     |

* The degree of TBA is indicated by the percentage of target thymocytes from young adult C3H-+/+ mice that were fluorescence positive after incubation with 1:2 diluted serum and FITC-GAMG. Anti-Thy-1.2 alloantisera gave >96% positive target thymocytes.

* Values are expressed as mean percent ± SE. Numbers of sera tested are in parentheses.

* One-half of the serum samples from 24-wk-old gld/gld and +/+ mice were from female donors.

* +/+ refers to wild-type allele (normal) at both the gld and lpr loci.

* Probability determined by Student's t test for independent means. NS, not significant at P < 0.05.
chromosome 1 between the Pep-3 and Lp loci. The chromosomal location of the lpr mutation is not known.

Beyond these basic similarities, C3H-gld and MRL-lpr mice have several fundamental differences: (a) C3H-gld mice have an extended postproliferative clinical course with an episode of regression of lymph node mass and survival extending to 1 yr of age. (b) C3H-gld mice have a substantial increase in the frequency of Thy-1-negative and sIg-negative (null) cells in their lymph nodes. (c) At the peak of primary lymphoproliferation, C3H-gld mice have major increases (>13-fold) of serum IgA and IgG2b. (d) Histologically, C3H-gld mice are essentially free of the profound perivascular lymphoid infiltrates routinely observed in the kidney, liver, and other organs of MRL-lpr mice. (e) In C5H-gld mice, the renal glomerulus and tubular apparatus is essentially normal at the age when MRL-lpr mice are dying with subacute immune complex glomerulonephritis (i.e., 4–6 mo). (f) Vascular disease, such as necrotizing arteritis and polyarteritis, seen commonly in MRL-lpr mice, is not found in C3H-gld mice. (g) C3H-gld mice develop moderately high levels of TBA by 20 wk of age. In contrast, MRL-lpr mice are essentially negative. (h) C3H-gld mice consistently develop an interstitial pneumonitis without major perivascular lymphoid infiltrations.

The need for animal models of spontaneous respiratory diseases, such as chronic interstitial pneumonitis (including hypersensitivity pneumonitis), has been discussed by Reid (30). Several distinct pulmonary diseases in man associated with aberrant immune functions, including autoimmunity, have been reviewed by Schatz et al. (31). Among these disorders are lymphocytic interstitial pneumonitis (often with coexistent Sjögren's syndrome) (32), immunoblastic lymphadenopathy (33), idiopathic interstitial pneumonitis with immune complexes (34), and lupus pneumonitis (35).

Interstitial lung disease has been experimentally induced in laboratory animals using both allogeneic soluble lung antigen (in strain C3H/He) (36) and preformed immune complexes (37). In addition to the severe chronic lupus nephritis characteristic of (NZB × NZW)F1 hybrid, Eisenberg et al. (38) identified a coexistent pulmonary interstitial disease that was considered immunologic in origin. Preliminary studies of lungs from C3H-gld mice have not implicated an extrinsic microbial factor. Serological studies have not detected an antiglomerular basement membrane-like factor by immunofluorescence. Direct immunofluorescence studies do not indicate significant immune complex (IC) deposition. In view of the above and the absence of damage to other tissues usually associated with IC disease (membranous glomerulonephritis, vasculitis), mutant gld mice may be useful in studies that explore alternative mechanisms of immunologically mediated pulmonary disease.

Genetic interactions may have a major influence on the pathogenesis and clinical course of gld- and lpr-induced autoimmune diseases. We have successfully transferred the mutant gene lpr to strain C3H/HeJ by multiple (>10) cross-intercross matings. Although MRL-lpr and C3H-lpr mice have similar latent periods and degree of generalized lymphadenopathy, the longevities of lpr mice on these two backgrounds are markedly different: the average lifespan of MRL-lpr is 5 mo compared with 11 mo for C3H-lpr mice. Thus, the genomic
background may have a significant effect on the expression of other mutant genes (such as glv) that affect the regulation of the immunologic apparatus. To evaluate more precisely the specificity of glv-determined immune aberrations (compared with lpr), we have developed glv and lpr congeneric lines sharing the common inbred backgrounds C3H/HeJ, C57BL/6J, and SJL/J.

The defective lipopolysaccharide response gene (Lps) present in strain C3H/HeJ (39) may have a specific influence on the expression of glv (as well as lpr). Both the glv and lpr mutant genes have been transferred to the closely related non-Lps-bearing (normal) strain C3HeB/FeJ. Parallel populations of homozygous glv mice with and without the Lps mutation are currently being followed in lifespan studies. Izui et al. (40) have demonstrated that the injection of the polyclonal B cell activator bacterial lipopolysaccharide is able to induce anti-DNA antibodies in most inbred strains of mice, including C3HeB/FeJ but not C3H/HeJ. We have determined that young adult C3H/HeJ-glv mice spontaneously produce high titers of anti-dsDNA antibody. Spontaneous polyclonal B cell activation (PBA) is a characteristic of mice with autoimmune disorders, such as Palmerston North (PN), NZB, (NZB × NZW)F1 , MRL lpr , and male BXSB, but not of mice from other, "normal" inbred strains (41, 42). The new mutant gene, glv, on inbred background C3H/HeJ, may be of value in determining whether PBA is a critical factor in the pathogenesis of spontaneous autoimmunity.

In view of the extreme hyperactive state of the B cell system in C3H-glv mice, one of the most intriguing findings in this study was the relative lack of significant damage to tissues that are commonly affected in IC disease, such as the renal glomerulus and blood vessels. The lack of cellular infiltrates in such organs as the kidney, liver, salivary glands, and pancreas is unique in comparison with other spontaneous murine models. The immunobiology of IC formation and subsequent events, including tissue deposition and reactivity of the host, is a complex process (43) that may become more fully understood in studies of C3H-glv mice.

In addition to their value in pathologic studies at the tissue level, single-gene mutations causing autoimmune, lymphoproliferative, and immunodeficiency disorders have been of value in studies of immunoregulatory networks (44). Deficiencies relating to the T cell regulatory lymphokine, interleukin 2 (IL-2), have recently been identified in lpr mutant mice (45, 46). It is generally accepted that normal cellular interactions depend in large part on the recognition of molecules expressed at the cell surface. Recently, the lymphocyte cell surface of lpr/lpr mice has been the focus of several studies. The uniqueness of the hyperplastic lymphoid cell has been defined by classic and novel antisera directed against cell surface antigens (19, 24), as well as a variety of lectins (20). In the present study, at the peak of primary lymphoproliferation (18–24 wk), the lymph nodes of C3H-glv mice had increased numbers of B cells (sixfold), T cells (15-fold), and a dramatic expansion (33-fold) of null (sIg−, Thy-1−) lymphocytes. A detailed examination of the lymphocyte surface phenotype, lymphocyte function, and production of and response to lymphokines, such as IL-2, will be critical to understand the pathogenesis of this disorder induced by the glv mutation.

It is becoming increasingly clear that the cellular basis for lupus is different among the major inbred and single-factor murine models (11, 12). To determine
the basic mechanisms leading to autoimmunity and consequential immunologic disease(s), the ability to isolate the genetic, viral, and environmental components is critical. Coisogenic and congeneic inbred strains differing at a single locus or chromosomal segment provide a powerful tool for studies of complex processes at the molecular level. The ability to manipulate Mendelian genes such as gld, on multiple inbred strain backgrounds or in combination with other mutations with known immune aberrations, such as bg, hr, Lps, nu, and xid (47), may enable us to dissect the factors involved in the regulation of normal immune responses and to define the common denominators in the pathogenesis of autoimmune diseases.

Summary

A newly discovered autosomal recessive mutation, generalized lymphoproliferative disease (gld), in the C3H/HeJ strain of mice, determines the development of early onset massive lymphoid hyperplasia with autoimmunity. Significant lymph node enlargement is apparent as early as 12 wk of age. By 20 wk, lymph nodes are 50-fold heavier than those of coisogenic C3H/HeJ-+/+ mice. There is a concomitant increase in the numbers of peripheral blood lymphocytes. Analysis of C3H-gld lymph node lymphocyte subsets by immunofluorescence indicates an increase in numbers of B cells, T cells, and null (Thy-1−, slg−) lymphocytes by 6-, 15-, and 33-fold compared with congeneric control mice. Serologically, gld/gld mice develop antinuclear antibodies (including anti-dsDNA), thymocyte-binding autoantibody, and hypergammaglobulinemia with major increases in several immunoglobulin isotypes. Mutant gld mice live only one-half as long as normal controls (12 and 23 mo, respectively). Interstitial pneumonitis was found in virtually all C3H-gld mice autopsied when moribund. Although immune complexes were detected in the glomerulus by immunofluorescence techniques, only 14% of the autopsied mice had significant lupus-like nephritis. Vascular disease was not found.

The pattern of early onset massive lymph node enlargement, hypergammaglobulinemia, and production of antinuclear autoantibodies resembles the basic abnormal phenotype induced by the lpr (lymphoproliferation) mutation. The mutations gld and lpr are not allelic. Linkage studies indicate that gld is located between Pep-3 and Lp on chromosome 1.

This new mutation adds another genetically well-defined model to the list of murine lymphoproliferative/autoimmune disorders that may be exploited to gain a clearer understanding of immunoregulatory defects and for identifying common pathogenetic factors involved in systemic autoimmune diseases.

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