Spontaneous Development of Plasmacytoid Tumors in Mice with Defective Fas-Fas Ligand Interactions

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Summary

B cell malignancies arise with increased frequency in aging individuals and in patients with genetic or acquired immunodeficiency (e.g., AIDS) or autoimmune diseases. The mechanisms of lymphomagenesis in these individuals are poorly understood. In this report we investigated the possibility that mutations at the Fas (lpr) and FasL (gld) loci, which prevent Fas-mediated apoptosis and cause an early onset benign lymphoid hyperplasia and autoimmunity, also predispose mice to malignant lymphomas later in life. Up to 6 mo of age, hyperplasia in lpr and gld mice results from the predominant accumulation of polyclonal T cell subsets and smaller numbers of polyclonal B cells and plasma cells. Here, we examined C3H-lpr, C3H-gld, and BALB-gld mice 6–15 mo of age for the emergence of clonal T and B cell populations and found that a significant proportion of aging mice exclusively developed B cell malignancies with many of the hallmarks of immunodeficiency-associated B lymphomas. By 1 yr of age, ~60% of BALB-gld and 30% of C3H-gld mice had monoclonal B cell populations that grew and metastasized in scid recipients but in most cases were rejected by immunocompetent mice. The tumors developed in a milieu greatly enriched for plasma cells, CD23+ B cells and immunodeficient memory T cells and variably depleted of B220− DN T cells. Growth factor–independent cell lines were established from five of the tumors. The majority of the tumors were CD23− and IgH isotype switched and a high proportion was CD5+ and dull Mac-1+. Considering their Ig secretion and morphology in vivo, most tumors were classified as malignant plasmacytoid lymphomas. The delayed development of the gld tumors indicated that genetic defects in addition to the Fas/Fasl mutations were necessary for malignant transformation. Interestingly, none of the tumors showed changes in the genomic organization of c-Myc but many had one or more somatically-acquired MuLV proviral integrations that were transmitted in scid passages and cell lines. Therefore, insertional mutagenesis may be a mechanism for transformation in gld B cells. Our panel of in vivo passaged and in vitro adapted gld lymphomas will be a valuable tool for the future identification of genetic abnormalities associated with B cell transformation in aging and autoimmune mice.

Key words: lpr • gld • Fas • Fas ligand • lymphoma

The recessive mutant genes, lpr and gld, map to the Fas and Fasl (ligand) loci, respectively (1, 2). In normal mice, Fas encodes a 45-kD cell surface receptor (Fas/CDD95/APO-1) belonging to the TNF/NGF receptor family and Fasl encodes a 40-kD type II membrane protein, FasL, homologous to members of the TNF family (1–5). In receptive cells, the aggregation of Fas receptors by FasL or anti-Fas mAb leads to the induction of cell death by apoptosis (6–8). Mice bearing the lpr mutation have a defect in the expression of Fas caused by the insertion of a retroviral transposon into the second intron of Fas that prevents normal transcription of the gene (1, 3). In gld mice, a point mutation in the COOH-terminal region of Fasl results in the expression of a non-functional form of Fasl on the cell surface (2, 4). Defective interactions between Fas and Fasl in mice homozygous for lpr or gld lead to indistinguishable, progressive diseases typified by profound lymphadenopathy, splenomegaly, high titers of circulating autoantibodies, hypergammaglobulinemia, strain-dependent systemic autoimmune disease and premature death (reviewed in reference 9). Recently, there have been several reports of pediatric patients with a variety of genetic mutations at the FAS locus and a spectrum of immune abnormalities closely resembling those of lpr and gld mice (10–14). The consistency of the immunologic defects associated with Fas mutations suggests a universal role for the Fas-associated cell deletion...
pathway in regulating lymphocyte survival and in preventing the accumulation of autoreactive B cells.

Between 1 and 6 mo of age, the spleens and lymph nodes of lpr and gld mice undergo progressive enlargement associated with the accumulation of two functionally unresponsive B220<sup>+</sup> T cell subsets not detected in normal mice. Lymphadenopathy results predominantly from the selective amassing of non-transformed B220<sup>-</sup>C.D4<sup>-</sup>C.D8<sup>-</sup> double negative (B220<sup>-</sup>DN) T cells (9, 15). The majority of these cells are derived from C.D8<sup>+</sup> precursors selected in the thymus on MHC class I Ag (16–19). The other B220<sup>-</sup> T cell subset is a minor one that expresses low levels of C.D4 and arises independently of MHC class I expression (16–20). As the B220<sup>+</sup> T cells accumulate, they dilute conventional C.D4<sup>+</sup> and C.D8<sup>+</sup> T cells and B cells and disrupt the normal architecture of the spleen and LN. Although normal T and B lymphocytes are reduced by proportion, their total numbers in LN are increased approximately 10-fold (21, 22). The expanded C.D4<sup>+</sup> and C.D8<sup>+</sup> T cell populations in 4–6-mo-old lpr and gld mice are greatly enriched for memory-like cells (9, 20–23). Similarly, the B cell population is enriched for cells with the phenotype of chronically activated B cells and for Ig-secreting cells (9, 24). The skewing of the T and B cell populations towards primed and activated cells is consistent with the demonstrated role of Fas-mediated apoptosis in regulating the survival of Ag-activated T cells and autoreactive B cells (25–29).

To further investigate the possibility that defective Fas-FasL interactions in lpr and gld mice lead to the accumulation of massive numbers of lymphocytes, there have been no reports of malignant T or B lineage tumors arising in these mice. In unpublished studies involving the transfer of spleen cells from BALB-gld mice with advanced disease into immunodeficient CB.17-sid mice, we observed that a significant number of recipients developed malignant B cell lymphomas of donor origin. To further investigate the possibility that gld mice spontaneously develop lymphomas that are masked by the preexisting lymphoproliferative disease, we surveyed groups of C.D4<sup>-</sup>- and BALB-gld mice of various ages for evidence of clonal, transformed lymphoid populations. These studies revealed that by 1 yr of age, 28% of C.D4<sup>-</sup>- gld mice and 57% of BALB-gld-gld mice had monoclonal outgrowths of B cells in spleen and LN. After transfer into sid recipients, the majority of these clonal B cell populations gave rise to metastatic plasmacytoid tumors. These findings provide evidence that in addition to limiting the accumulation of polyclonally activated and autoreactive lymphocytes, normal Fas-FasL interactions also prevent the development of B cell neoplasms.

### Materials and Methods

Mice. All mice were bred and maintained at PerImmune, Rockville, MD; National Cancer Institute contract NCI-CB-710-85. C.D4<sup>-</sup>-lpr/lpr (C.D4<sup>-</sup>-lpr) and C.D4<sup>-</sup>-gld/gld (C.D4<sup>-</sup>-gld) mice were bred from breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME). BALB/c-gld/gld (BALB-gld) mice were bred in our colonies from C.D4<sup>-</sup>-gld mice backcrossed for 15 generations to BALB/cAnPt mice. C.D4<sup>-</sup>-sid/sid mice breeding pairs were obtained from Sprague Dawley (San Diego, CA) and C.B-17-sid/sid breeding pairs were the gift of Dr. D. Hilbert (NCl, NIH, Bethesda, MD). For tumor transfer studies, sid recipients were injected i.p. with 1 × 10<sup>7</sup> donor spleen or LN cells. Tumor-bearing mice were palpated weekly and killed 2–6 wk post-transplant before they became moribund.

Characterization of Tumors. Lymphoid tissues from mice bearing primary tumors or from sid mice with tumor transplants were processed for histology, FACScan<sup>®</sup> analyses, and in vitro culture. Tumor cells were viably frozen and cell pellets were snap frozen for DNA and RNA extraction. Cell lines were established from spleen or LN cells from sid mice with tumor transplants. These lines were maintained in complete RPMI 1640 with 10% fetal bovine serum.

DNA Analyses. High MW DNA was isolated, digested with EcoRI, HindIII, Hpal or PvuII (Boehringer Mannheim, Indianapolis, IN), separated on 0.7% agarose gels, and blotted onto N.ytran membranes (Schleicher & Schuell, Keene, N.H.) using established techniques. Probes used included J<sub>b</sub>, a 1.96-kb BamHI-EcoRI fragment of the BALB/c germline J<sub>b</sub> region (30); IVS, a 1.1-kb EcoRI-Xba fragment of the BALB/c germline C<sub>b</sub> region (31); CT<sub>µ</sub>, a 618-bp BamHI-EcoRI fragment containing the J<sub>µ</sub> and C regions of 86TI (32), a cDNA clone for the β-chain of the T cell receptor; pEco, a 400-bp SmaI fragment derived from an AKR ecotropic virus env gene (33); and pvt, a 1.4-kb EcoRI fragment of the germline P<sub>vt</sub> (34). The c-Myc probe was a 1.3-kb fragment containing exon 2 and exon 3 (gift from Dr. K. Huppi, NCI, NIH, Bethesda, MD). The probes were 32<sup>P</sup>-labeled by nick translation (Lofstrand, Rockville, MD). Washings of Southern blots were done to a final stringency of 0.1× SSC, 0.5% SDS at 65°C. FACScan<sup>®</sup> Analyses. Single cell suspensions of spleen and LN were prepared, blocked with anti-CD32 mAb, and stained as described previously (15). Analyses were performed on a FACScan<sup>®</sup> (Becton Dickinson, Sunnyvale, CA). Dead cells were eliminated from the analysis by propidium iodide gating. Abs used for staining included various combinations of T-lymphocytic (T-C)-labeled anti-CD45(B220), anti-CD8 (Caltag, San Francisco, CA), PE-labeled mAb specific for CD4, IgL<sub>k</sub>, IgL<sub>λ</sub>, CD45(B220), CD8, CD32, CD19, and CD95(Fas) and FITC-labeled mAb specific for TCR-α/β, Th8, CD11b (Mac-1), CD23, CD80 (B7-1), CD86 (B7-2), 6C3 (Ly-6C), CD43, 1-A<sub>5</sub>, 1-A<sub>4</sub>, CD5, IgM, IgG1, 1G2a, and IgG3 (PharMingen, San Diego, CA).

Preparation of Tissues for Histology. Tissues were fixed in modified Tulleyesniczky's solution and processed at American Histolab, Gathersburg, MD.

Quantitation of A n-t-dsDNA. A uantitatives in Sera. 96-well flat bottomed microtiter plates were coated with 10 μg/ml methylated BSA (Sigma Chemical Co., St. Louis, MO) in carbonate buffer (pH 9.6) overnight at 4°C. After washing with PBS/0.05% Tween 20, the plates were incubated with 10 μg/ml calf thymus DNA in carbonate buffer overnight at 4°C. The plates were blocked with dilution buffer containing 2% PEG 8000, 1% gelatine, 0.05% Tween 20, 1% BSA in PBS for 30 min. at 37°C. Serum samples

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1<sup>1</sup>Abbreviations used in this paper: ALPS, autoimmune lymphoproliferative syndrome; DN, double negative; IL, immunodeficiency-associated lymphomas; MAIDS, mouse AIDS; RT, room temperature.
were diluted twofold starting at 1:500 in diluting buffer, added to the plates, and incubated for 2 h at 37°C. Anti-DNA Ab binding was detected with biotinylated anti-mouse IgG (Southern Biotechnology) and POD-labeled streptavidin using ABTS as the substrate.

Quantitation of Serum Ig Counts. Levels of IgG, IgG1, IgG2a, IgG2b, IgG3, and IgM in sera were determined by ELISA. ELISA plates were coated overnight at 4°C with 50 μl of goat antibodies specific for mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 diluted to 5 μg/ml or 50 μg/ml (IgG3) in carbonate buffer (KPL, Gaithersburg, MD). After washing the plates were blocked with 200 μl of SuperBlock (Pierce Chemical Co., Rockford, IL) for 20 min at room temperature (RT). Ig standards and serial dilutions of mouse sera (50 μl) in blocking buffer were added and the plates were incubated for 2 h at RT. After washing the plates with PBS-Tween, 100 μl of the following detecting mAb labeled directly with HRP or AKP were added to the appropriate wells. Anti-IgG2a (R19-16) 1:1,000, anti-IgG2b (R12-13) 1:2,000, anti-IgG3 (R5-60.2) 1:1,000, and anti-IgM (R19-15) 1:1,000 (PharMingen). The plates were incubated for 1.5 h at RT then washed with PBS-Tween. The HRP or AKP substrates (KPL, Gaithersburg, MD) were added, the reaction was stopped and the plates were read.

Results

Evidence for the Outgrowth of Clonal B Cell Populations in 11-15-mo-old BALB-gld and C3H-gld Mice. To further investigate the possibility that defects in the Fas-FasL-mediated cell death pathway may predispose mice to the development of B cell lymphomas, we surveyed groups of 4–8-mo-old and 9–16-mo-old BALB-immunodeficient mice for evidence of clonal expansion of cells with rearranged IgH, IgL, or TCR genes, and fresh or via-}

Table 1. Outgrowth of Clonal B Cell Populations in C3H-1pr, C3H-gld, and BALB-gld Mice

| Strain       | Number of mice | Mono- or biclonal | Oligoclonal | N one |
|--------------|----------------|-------------------|-------------|-------|
| BALB-gld     | 4–8            | 12                | 2           | 3     | 7     |
|              | 9–15           | 46                | 26          | 13    | 4     |
| BALB-+/+     | 10–21          | 22                | 0           | 0     | 22    |
| C3H-gld      | 4–8            | 9                 | 0           | 0     | 9     |
|              | 11–15          | 68                | 19          | 10    | 39    |
| C3H-1pr      | 11–15          | 25                | 8           | 4     | 14    |
| C3H-++       | 11–15          | 25                | 0           | 0     | 25    |

*Spleen and LN cell DNA with ≥4 prominent rearranged IgH and IgL bands.

†Spleen and LN cell DNA with ≥4 rearranged IgH and IgL bands.

§No detectable clonal rearrangements.

Of the 39 samples with clonal IgH and IgL rearrangements, 26 (67%) had between one to three prominent rearranged IgH and IgL bands common to spleen and LN suggesting the selective outgrowth of one or two B cell clones. Fig. 1 shows typical examples of members of this group. Except for sample 540, only data for LN DNA is shown,
if the monoclonal/biclonal B cell populations accumulating with age in the BALB-gld and C3H-gld mice were transplantable, fresh or viably frozen spleen or LN cells were injected intraperitoneally into immunodeficient scid recipients. Of 23 suspected tumors inoculated, twenty gave rise to metastatic monoclonal B lineage tumors 3–8 wk after inoculation. As shown in Figs. 1 and 2, each tumor transplant had identically rearranged IgH and IgL bands to the clonal B cell populations in the primary inoculum. In one C3H-gld mouse, 217, at least two B cell clones were present originally but only one grew out consistently in scid mice (Fig. 2). The tumors showed some variations in their growth patterns in adoptive transfers. The C3H-gld tumor 355 grew very aggressively in all lymphoid organs. All of the other tumors grew rapidly in the spleen, mesentery, mediastinal and mesenteric LN and more slowly in peripheral LN. The majority of the tumors metastasized to the liver, lungs, and kidneys. Some tumors, (e.g., C3H-gld tumors 142, 217, and 221 and the BALB-gld tumor 540) also grew in the ovaries and uterus. Only the C3H-gld tumors 217 and 205 induced copious production of ascites. In contrast, there was no selective outgrowth of B cells in scid mice injected with spleen or LN cells from gld or 1/1 mice with no evidence of clonal B cell populations (data not shown). The C3H-gld tumors 142, 205, 217, 355, and 362 were injected i.p. or s.c. into 6–8-wk-old C3H 1/1, -gld, and -lpr recipients and the BALB-gld tumors 311 and 208 were injected i.p. or s.c. into 6–8-wk-old BALB 1/1 and -gld mice to determine if they would grow in immunocompetent mice or have a growth advantage in gld recipients. The cells used for injection were grown in scid mice and were highly enriched for tumor cells. Tumor growth was assessed histologically, by FACS, and by Southern blot analysis. Tumor 355 grew very aggressively in the peritoneal cavity, spleen, and peripheral LN in all three groups of recipients and killed the mice within 2 weeks of inoculation. Tumor 142 grew in the ovaries of 1/5 C3H-gld recipients but was not detected in C3H-lpr or C3H 1/1 mice 2 mo after injection. None of the remaining tumors grew in, or were recoverable from, any of the recipients 2–6 mo after inoculation. To determine if the tumors had a growth advantage in older mice with advanced lymphoproliferative disease, tumors 217 and 205 were injected i.p. into groups of 7 mo
old lpr mice and the mice were killed 2 mo later. No tumor growth was detected in any of the recipients (data not shown).

Age-related Changes in the Cellular Composition of Lymphoid Organs in gld Mice. The BALB-gld mice used in this study have not been described previously. These mice developed progressive lymphoproliferative disease closely resembling that of C3H-gld mice in terms of time of onset, severity, and pathology. At 4–5 mo of age, when lymphadenopathy and splenomegaly were well established and comparable in both strains, some consistent but minor strain-related differences were observed in the proportions of the various T cell subsets. As shown in Fig. 4, B220+ DN T cells predominated in the LN of both strains of mice at 4–5 mo of age but the BALB-gld mice had lower proportions of this subset and higher proportions of CD4+ T cells, CD8+ T cells, and B cells than the C3H-gld mice. Between 6 mo and ~1 yr of age, a previously unreported redistribution of lymphocyte subsets occurred in the LN of both BALB-gld and C3H-gld mice. This was more apparent in the C3H-gld mice and was characterized by a selective decrease in the proportions of B220+ DN T cells from 79.5 ± 1.4% to 35.1 ± 2.1%, and a three- to fourfold increase in the proportions of CD4+ T cells, CD8+ T cells and B cells (Fig. 4). A small 1.6-fold increase in the proportion of CD4+B220+ T cells also was observed (Fig. 4). Spleens from aging C3H-gld mice and LN and spleens from aging BALB-gld mice showed a similar pattern of changes in the distribution of T cell subsets (Fig. 4 and data not shown). These changes in cellular composition were not

Figure 3. Clonal B cell outgrowths are not detected in the spleens of 12–15-mo-old Balb/c1/1 mice. Panels show Southern blot analyses of genomic DNA for IgH (top) and IgL (bottom) gene rearrangements. DNA was isolated from the spleens of 12–15-mo-old Balb/c1/1 (lanes 1–11) or from Balb/c1/1 kidney (lane 12). GL indicates the position of the germ-line IgH and IgL bands. Data are representative of 25 mice analyzed.

Figure 4. Age-related changes in the distribution of T and B cell subsets in the LN of C3H-gld and BALB-gld mice. The percentage of each lymphocyte subset (identified in top right hand corner) was determined by 3-color FACS® analysis of LN cells from individual BALB-gld and C3H-gld mice aged 4–5 mo, 7–10 mo, and >11 mo. Data is shown for all mice >11 mo and mice >11 mo with tumors. The proportions of B220+ DN T cells were determined by staining cells with PE-labeled anti-CD4 and anti-CD8, Tri-color-conjugated anti-CD45(B220), and FITC-labeled anti-TCR-α/β. The histograms represent the mean plus SE of values for 11 to 68 C3H-gld mice (top six panels) and 11–23 BALB-gld mice (bottom six panels).
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accompanied by a reduction in LN or spleen size or cellularity and did not correlate with the development of B lineage tumors (Fig. 4 and data not shown). Among the B cells, there was an age-related increase in the proportions and numbers of kappa B220+CD19+ThB+CD5−CD23−cells. By ~1 yr of age, the majority of the B cells in LN and spleen were of this phenotype (Fig. 5). Among these cells, a subset also expressed low to intermediate levels of Mac-1 (Figs. 4 and 5). In LN, the Mac-1+ subset represented fewer than 0.5% of cells at 4–5 mo of age and by ~1 yr of age had increased to 2.7 ± 0.9% in C3H-gld and 7.5 ± 1.8% in BALB-gld mice. Between 4 and ~12 mo of age, the proportions of Mac-1+ B cells in spleen increased from 1.6 ± 0.3% to 5.0 ± 0.4% for C3H-gld mice and from 2.6 ± 0.5% to 9.8 ± 1.4% for BALB-gld mice. In contrast, this subset represented fewer than 3% of the cells in the spleens of 11–15-mo-old C3H+/+ and BALB+/+ mice (data not shown). Although the accumulation of CD23+ Mac-1+ B cells was greatest in tumor-bearing mice, animals lacking tumors also had significantly increased proportions of these cells (Figs. 4 and 5 and data not shown).

Comparisons of the Phenotypes of the Primary Tumors and scid-propagated Tumors. Among the tumor-bearing mice, Ig+ B cells rarely exceeded 10–20% of the total population in LN and spleen and the tumor population was not readily identified by a unique phenotype or selective increase in size. Therefore, accurate phenotyping and morphologic classification of the tumors was dependent on their selective outgrowth in scid transplants. This is illustrated in Fig. 6 for tumor 217 which is representative of the majority of the tumors isolated. The LN of the mouse bearing the primary tumor 217. The adjacent panels show corresponding data for LN cells from C3H-scid mice bearing the primary (center) and secondary (right) 217 transplants. Cells were stained with the reagents indicated and analyzed on the FACScan. The values inside each quadrant represent the percentage of reactive cells.
Table 2. Tumor Surface and Secreted Ig Isotypes

| Tumor     | Surface Ig* | Secreted Ig† | MYC/Pvt 1 rearrangements‡ |
|-----------|-------------|--------------|----------------------------|
| C3H-gld   |             |              |                            |
| 142       | IgG2a       | IgG2a        | ND                         |
| 205       | Null        | IgG2a        | ND                         |
| 217       | IgG3        | IgG3         | ND                         |
| 221       | Null        | IgA          | ND                         |
| 223       | IgG3        | IgG3         | ND                         |
| 355       | Null        | IgG2a        | ND                         |
| 362       | Null        | IgG3         | ND                         |
| BALB-gld  |             |              |                            |
| 208       | IgG1        | IgG1         | ND                         |
| 311       | Null        | IgG3         | ND                         |
| 329       | IgM         | IgM          | ND                         |
| 421       | IgM         | IgM          | ND                         |
| 424       | IgA         | IgA          | ND                         |
| 425       | IgG2a       | IgG2a        | ND                         |
| 426       | IgA         | IgA          | ND                         |
| 536       | IgA         | IgA          | ND                         |
| 540       | IgG2a       | IgG2a        | ND                         |

*Surface Ig expression on s1d-passaged tumor cells determined by FACS® analysis.
†Isotype of secreted Ig in sera from tumor-bearing s1d mice determined by ELISA assay.
‡Rearrangement of MYC and PVT1 loci determined by Southern blot analysis of genomic DNA from s1d-passaged tumors.
ND, not detected. Rearrangements of MYC or PVT1 were not detected.

CD19, CD80, and CD86 and levels of ThB ranging from low to high. Only 355 was Fas−. Most of the tumors were CD23− and expressed low to intermediate levels of CD5 and low levels of MAc-1. Five tumors, 142, 205, 355, 362, and 311, were consistently slg− and the remainder had low to intermediate levels of IgH and IgL chains. With the exception of 142 which was λ+, all slg+ tumors expressed kappa L chains. A variety of IgH isotypes were expressed including IgM, IgG1, IgG2a, IgG3, and IgA (Table 2). Sera from the majority of the s1d mice transplanted with Ig+ C3H-gld and BALB-gld tumors had high titers of Ig of the same isotype expressed on the tumor (Table 2). The surface Ig+ tumors growing in s1d mice also secreted Ig of a single isotype (Table 2). Notably, none of the sera reacted with ds-DNA (Table 2). In many instances, the primary tumors also appeared to be secreting Ig as one or two gamma spikes were detected in electrophoreses of sera from 66% (19/29) of tumor-bearing mice (data not shown). Histologically, the tumor populations in the primary s1d tumors were a mixture of immunoblasts, plasmacytoid cells and plasma cells.

Surprisingly, 40% of the LN cells in the primary s1d transplant of 217 were T cells (28% TCR-α/β+CD8+ T cells and 12% TCR-α/β+CD4+ T cells) and significantly more T cells were recovered than were present in the original inoculum. The proliferation of T cells in the first s1d transfer was observed with many of the C3H-gld and BALB-gld tumors and was not always skewed towards CD8+ T cells. Analyses of TCR-β chain gene rearrangements showed no evidence of clonality among the T cell populations in any of the s1d recipients (data not shown). Of note, there was no significant outgrowth of B220+ DN T cells or B220+CD4+ T cells in any of the s1d mice inoculated with tumor-bearing populations.

In secondary s1d transfers with LN cells from the primary s1d recipients, clonal descendants of the 217 tumor cells predominated and T cells represented fewer than 3% of the cells (Fig. 6). The tumor population was enriched further for plasmacytoid cells and plasma cells as illustrated by high titers of serum IgG3 and changes in phenotype and morphology. The majority of the cells remained IgG3+CD45+CD5+ThB+CD8−CD86−Fas+CD19− and a significant proportion downregulated their expression of surface Ig and switched from CD45(B220) expression to a lower MW CD45 isoform (Fig. 6 and data not shown). This skewing of the tumor population towards more differentiated plasmacytoid cells and plasma cells in secondary and subsequent s1d transfers was a hallmark of the majority of the tumors arising in C3H-gld and BALB-gld mice.

Establishment and Phenotype of Tumor Cell Lines. Among the 19 tumors that grew in s1d recipients, 5 (142, 205, 217, 355, and 311) have been adapted successfully to culture. All of the tumors propagated in s1d mice survived for at least 2-3 wk in culture but were difficult to maintain long-term. Only 355 grew aggressively and independently from the beginning. Initially, tumors 142, 205, 217, and 311 were highly dependent on adherent stromal cell populations for their survival and these could not be substituted with IL-6. The tumor cells adhered tightly to the stromal cells and had to be trypsinized to be passaged. Subsequently, stromal cell–independent sublines were established for all four lines. These lines grew both in large clusters and as weakly adherent monolayers. Analyses of IgH and IgL gene rearrangements confirmed that each cell line was clonally related to the s1d tumor from which it was established (Figs. 1 and 2). The tumor population was enriched further for plasmacytoid cells and plasma cells as illustrated by high titers of serum IgG3 and changes in phenotype and morphology. The majority of the cells remained IgG3+CD45+CD5+ThB+CD8−CD86−Fas+CD19− and a significant proportion downregulated their expression of surface Ig and switched from CD45(B220) expression to a lower MW CD45 isoform (Fig. 6 and data not shown). This skewing of the tumor population towards more differentiated plasmacytoid cells and plasma cells in secondary and subsequent s1d transfers was a hallmark of the majority of the tumors arising in C3H-gld and BALB-gld mice.

Histopathologic changes in aging C3H-gld and BALB-gld Mice. The first organs to be affected in C3H-gld and BALB-gld mice were the lymph nodes, particularly the
subcutaneous ones. Normal LN architecture was lost as the B220+ DN T cells selectively accumulated. By ~4 mo of age, sheets of homogeneous medium-sized DN T cells with characteristically stippled chromatin totally replaced the cortex and expanded the medullary cords so that the sinusoids were effaced (Fig. 8 A). At the same time, the splenic white pulp became greatly enlarged and cells morphologically resembling the B220+ DN T cells occupied both the peripheriolar sheath and the mantle zone (data not shown). In both the LN and spleen, plasmacytoid cells were present in variable numbers but they represented a small minority of cells. In the lung, peripheriolar cuffing by lymphoid cells was moderately advanced, but the kidneys and liver were unaffected at this time (data not shown).

Beyond 6 mo of age, the LN and spleen underwent further changes in cellular composition and cellular infiltration of nonlymphoid tissues was more widespread. These changes occurred in both tumor-bearing mice and mice without apparent tumors. In LN and spleen, the cells became progressively less densely packed and the stromal elements were more apparent. The homogeneous DN T cell population was replaced increasingly by small lymphocytes, immunoblasts, plasmacytoid cells, plasmablasts, and plasma cells. Consistent with a previous report by Jacobson et al. (35), the plasma cells in spleen characteristically accumulated around the central arteriole in the peripheriolar lymphoid sheaths. Infiltration, mainly by plasmacytoid cells, became increasingly severe in the lungs, portal areas of the liver, and renal medulla. Fig. 8 B shows typical cellular changes and plasma cell infiltrates in the LN of a 12-mo-old C3H-gld mouse with no detectable tumor.

The increased presence of the activated B cell populations and plasma cells, together with the underlying T cell accumulation greatly complicated the morphologic classification of the primary tumors. This difficulty is illustrated well by the prototypic tumor 217. For this tumor, there was moderate splenic and hepatic, and more severe renal infiltration. However, as shown in Fig. 8 C and D, the cells...
were morphologically heterogeneous making it difficult to identify the tumor population. In the first and all subsequent passages of 217 in scid mice, there was extensive tumor infiltration of the lymphoid organs, lungs, liver, mesentery, perirenal fat, ovaries, and uterus and to a lesser extent kidneys. Fig. 8 E illustrates the intrauterine infiltration in the first scid passage. In the primary passage of 217 and most of the other C3H-gld and BALB-gld tumors, the infiltrating cells were a mixture of donor T cells and tumor cells but in the subsequent passages the infiltrates were composed almost exclusively of monoclonal tumor cells (Fig. 5 and data not shown). In all passages, the 217 tumor population was morphologically heterogeneous and included immunoblasts, plasmablasts and plasma cells in varying proportions (Fig. 8 F). All of the BALB-gld and most of the C3H-gld tumors closely resembled 217 in their patterns of growth and morphology. The exceptions among the C3H-gld tumors were 355, 205, and 142. Whereas these clearly were mature B cells by phenotype and Ig secretion, they were less differentiated morphologically. In summary, even though morphologic identification of the primary C3H-gld and BALB-gld tumors was difficult, their clonal descendants that proliferated in scid recipients almost universally were skewed towards the late stages of B cell differentiation and were classified as malignant plasmacytoid lymphomas.

C3H-gld and BALB-gld Tumors Have Somatically Acquired, Clonal MuLV Proviral Integrations. The transformation of mature B lineage cells frequently involves multiple genetic events including the dysregulated expression of cellular protooncogenes. For example, plasmacytomagenesis often is associated with dysregulated expression of c-Myc caused by the translocation of this gene to the switch regions of the IgH locus or by rearrangement of the Pvt1 locus (34, 36). Abnormal expression of oncogenes also can occur following the insertion of infectious endogenous retroviruses into the genome (37). To determine the potential involvement of either of these mechanisms in the development of the gld tumors, DNA from the primary tumors, scid-passaged tumors and tumor cell lines digested with PvuII and probed with a 32P-labeled ecotropic MuLV probe. DNA from C3H-gld kidney shows the position of the single hybridizing germline (GL) band present in normal C3H cells.
of c-myc translocation, or amplification or disruption of the PviT locus (data not shown).

Both C3H and BALB/c mice have single germline copies of endogenous infectious ecotropic MuLV (Figs. 9 and 10). In contrast, a high proportion of the C3H-gld and BALB-gld tumors had somatically acquired MuLV proviral integrations. As shown in Fig. 9, the transplantable C3H-gld tumors 142, 205, 355, 221, 223, and 362 had between 2 and 13 viral integrations in the primary tumor. 217 was the only primary tumor with no evidence of newly acquired proviral integrations. A different pattern of viral integrations was observed for the BALB-gld mice (Fig. 10). Among 17 monoclonal transplantable tumors surveyed, seven had newly acquired proviral integrations in both the primary and c-sod-propagated populations (e.g., 425 and 540), seven had integrations in the transplanted but not the primary tumor (e.g., 311 and 536), and three had no detectable new integrations (e.g., 329). In general, the primary BALB-gld tumors had many fewer somatically acquired proviral insertions than the C3H-gld tumors (Figs. 9 and 10). This difference is most likely explained by known differences between BALB/c and C3H mice in the genetic loci that regulate the spread of N-tropic ecotropic virus with BALB/c mice being restrictive and C3H mice permissive (38). Most of the bands in the primary C3H-gld and BALB-gld tumors also were present in the c-sod transplants and in the in vitro cell lines indicating that the majority of the proviral integrations are stable. In some cases new bands arose in the c-sod transplants and the cell lines. This is particularly evident in the c-sod transplants of 217 and 536 and in the 311 cell line (Figs. 9, 10). Although these new integrations clearly are not involved in tumor induction, they may be important for tumor progression or adaptation to culture. The great variation in the sizes of the viral bands indicate that there are few common integration sites among the tumors. It should be noted that somatically acquired ecotropic virus integrations were not detected in DNA from young gld mice or tumor-free aged gld mice (data not shown).

**Discussion**

This study was undertaken to examine the effects of the life-long absence of a functional Fas-FasL-mediated cell deletion pathway on spontaneous lymphomagenesis. We showed that a mutant, nonfunctional FasL significantly accelerated the onset and increased the incidence of lymphomas in C3H and BALB mice. The tumors developed in a milieu greatly enriched for plasma cells, CD23– B cells and memory-like CD4+ and CD8+ T cells and variably depleted of B220+ DN T cells. The lymphomas were restricted to the B cell lineage, were skewed towards the terminal stages of B cell differentiation, were transplantable in immunodeficient mice, and hence were classified as malignant plasmacytoid lymphomas. The majority of the tumors were CD23– and IgG isotype switched and a high proportion were CD5– M ac-1 dull. None of the tumors showed changes in the genomic organization of c-myc.

The age-related accumulation of a putatively primed or activated IgM+CD23–CD5– cell subset unrelated to CD23– B-1a or B-1b cells has been reported previously for MRL-lpr, B6-lpr, and B6-gld mice (24). Our studies show that a similar B cell population accrues in aging C3H-gld and BALB-gld mice, and that in these strains a subset of the cells also express low levels of M ac-1. The accumulation of CD23– cells in lpr and gld mice may be a consequence of chronic B cell activation and/or increased longevity stemming from the Fas-FasL defect. If the CD23– B cells are long-lived, they may be at increased risk of transformation, and in this regard resemble the naturally long-lived B-1a and B-1b cells that give rise to lymphomas in aging NZB mice (39). The development of gld tumors that phenotypically resembled isotype-switched CD23–B2 cells, CD23–CD5–M ac-1– B-1a cells, and CD23–CD5–M ac-1– B-1b cells implies that each of these B cell subsets may be susceptible to transformation in gld mice.

There is compelling evidence that chronic inflammatory diseases or infections resulting in persistent lymphoproliferation may be conditioning events in the development of B cell malignancies (40–42). As one example, there is a strong correlation between infection with Helicobacter pylori and the development of B lineage gastric lymphomas (43, 44). Although chronic infections are unlikely to be a driving force in the development of tumors in Fas-Fasl-deficient mice, self antigens may be responsible for chronic B cell activation. In support of this proposal, lymphoma-bearing TCR-β/δ-deficient lpr mice had high titers of circulating anti-mouse IgG autoantibodies (45). In the present study, none of the gld tumors secreted detectable levels of anti-dsDNA autoantibodies, but a role for other autoantigens in promoting chronic B cell stimulation has not been eliminated.

**Figure 10.** Somatically-acquired clonal MuLV proviral integrations in BALB-gld tumors. Southern blot analysis of PvuII-digested genomic DNA from BALB-gld primary tumors, c-sod transplants and the 311 cell line. The blot was hybridized with a 32P-labeled ecotropic MuLV probe. Control DNA samples from BALB-gld and C.B-17-sod kidneys show the position of the germine (GL) band.
Previously, we and others reported an age-related accu-

mulation in lpr and gld mice of memory-like CD4+ and

CD8+ T cells with the capacity to secrete high levels of IL-4, IL-10, IFN-γ, and TNF-α (20–23). These cytokines, with their well-established effects on B cell survival, proliferation and differentiation (46–49), also may promote or enhance B cell activation and contribute to the prolonged survival and accumulation of plasma cells. Paradoxically, the accumulation of memory cells in lpr and gld mice is associated with defects in the capacity of T cells to proliferate and secrete IL-2 in vitro in response to a wide variety of mitogens, and superantigens (9, 50). Skewing of the T cell pop-

ulation towards memory cells and losses in mitogenic responses and IL-2 production also occurred in aging hu-

mans and normal 2–3 yr-old mice (51–53). In aged +/- mice, the memory T cell population had decreased expres-

sion of Fas and a defect in FasL-induced apoptosis (53).

Moreover, overexpression of Fas in CD2-Fas-transgenic mice prevented the age-related accumulation of immuno-
deficient memory cells and also delayed thymic involution (53). These observations suggest that T cell senescence is associated with defective Fas-mediated signaling and this may explain the apparent acceleration of immune senes-
cence in lpr and gld mice.

Our finding that the majority of the gld B cell tumors were rejected by young +/-, lpr and gld mice, implies that the tumors were immunogenic for immunocompetent mice and that rejection was not dependent on a functional Fas-mediated apoptosis pathway. Parallel results were obtained in mice made T cell-deficient by inactiva-
tion of both TCR-β and TCR-δ genes. These mice nor-
mally develop IgM+ B cell lymphomas with low incidence but tumor development was increased greatly if the mice also were homozygous for lpr (45). In contrast to our sys-
tem, the tumors in the T cell-deficient lpr mice developed rapidly and ~60% of the mice had tumors by 7 mo of age (45). Interestingly, lymphomagenesis in the lpr mice was prevented if the mice had either TCR-α/β+ or TCR-γ/δ+ T cells, implying that the Tumor cells were immuno-
genic and that in young immunocompetent mice, each T cell type was able to delete the tumor cells by a Fas-inde-

pendent pathway (45). This study and ours suggest that chronic activation and enhanced survival of B cells together with defective immune surveillance contribute to acceler-
ated lymphomagenesis in Fas-Fasl-deficient mice.

A strong correlation between immunodeficiency and B cell lymphomagenesis has been reported previously in hu-

mans and mice (40–42, 54, 55). In humans, immunodeficiency resulting from treatment with immunosuppressive drugs or secondary to infection with HIV greatly increases the risk of B cell lymphoma development (41, 54, 55). Similarly, patients with congenital or age-related immuno-
deficiencies, with a variety of autoimmune diseases, or with angioblastic lymphoproliferative disorders have an in-

creased risk of developing B lineage tumors (41, 54, 56).

Like the gld tumors, human immunodeficiency-associated lymphomas (IAL) also can retain their immunogenicity.

For example, IAL in organ transplant patients have been

reported to spontaneously regress when immunosuppres-
sive therapy is discontinued (41, 55).

Among mice, B lymphomas also develop with high fre-

quency in C57BL/6 mice infected with LP-BM5 murine leukemia viruses which cause an acquired immunodefi-
ciency syndrome designated mouse AIDS (MAIDS; 57). These aggressive IAL also can be propagated readily in sdd mice but are rejected by immunocompetent mice (58). In another system involving C57BL/KaLwRij mice, a murine model of multiple myeloma, plasmacytomagenesis corre-
lated strongly with an age-related T cell immunodeficiency (50). Because the gld B lymphomas share many of the hallmark of mouse and human IAL, we propose that they represent a novel subset of IAL that develops as a result of the complex and cumulative effects on the immune system of defective Fas-Fasl interactions. These effects may in-
clude chronic Ag-driven B cell activation, the ready avail-

ability of T cell help and growth factors, the loss of a major elimination pathway for activated B cells, and an age-related deficit in T cell–dependent immune surveillance.

The increased risk of B cell lymphomagenesis associated with Fas and Fasl mutations is not restricted to mice. Re-
cently, a family was described in which two out of four members with germline FAS mutations and autoimmune lymphoproliferative syndrome (ALPS) were diagnosed with B cell lymphomas at 25 yr of age (61). In contrast, none of the reported pediatric cases of ALPS with germline FAS mutations has a history of lymphoma (10–14). Al-

though the number of cases is small, the delayed develop-
ment of B cell tumors in the patients is consistent with our findings in lpr and gld mice and implies that progression to malignancy is dependent on the accumulation of additional mutations in DNA. Other evidence for a possible associa-
tion between FAS mutations and B cell malignancy comes from a recent study of patients with multiple myeloma in which FAS mutations were detected in 10% of tumor-con-
taining bone marrow aspirates (62). Although we observed nonlymphoid tumors only rarely in lpr and gld mice, two of four ALPS patients described by Drappa et al. (14) de-
veloped nonlymphoid tumors in adulthood. It is not clear if these tumors arose as a result of treatment with cytotoxic drugs or infection with hepatitis virus or if FAS mutations generally increase the risk of neoplasia.

Little is known about the sites or mechanisms of muta-
tion in IAL. Experimentally-induced plasmacytomas in BALB/c mice consistently have cMYc-activating t(12;15) or t(6;15) translocations that are believed to be vital for the transformation process (34, 36). In contrast, our primary or in vivo-induced BALB-gld and C3H-gld plasmacytoid tumors, which often evolved into plasmacytomas in sdd passages, had no evidence of cMYc or PVT1 rearrangement or amplification suggesting that novel genetic mutations may substi-
tute for cMYc dysregulation in these tumors. Similarly, cMYc translocations were not detected in the multiple myelomas arising in the C57BL/KaLwRij mice or in the MAIDS B cell lymphomas (58–60). In other experimental mouse models of lymphomagenesis, murine leukemia viruses have been found to induce neoplasms by integrating into the
host DNA and mutating or transcriptionally activating flanking genes (37). Our observation that a high proportion of the gld tumors had one or more somatically acquired proviral integrations that were stably transmitted in sis passages and cell lines, raises the possibility that insertional mutagenesis may be a mechanism of transformation in gld IA/L. It is not known if interference with the Fas-mediated cell death pathway only serves to increase the pool of targets for transformation and to protect the tumors cells from Fas-mediated apoptosis, or if mutations in Fas or Fasl cooperate with other mutations. In this regard, it recently was reported that the lpr mutation accelerated lymphomagenesis in Eμ L-myc transgenic mice but not in Eμ Pim-1 transgenic mice (63,64), indicating that under some circumstances cooperation may occur.

Both BALB/c/+ and C3H/+/ mice have been reported to develop B lineage tumors, but these generally were not detected until the mice were 2–3 yr of age (65, 66). In addition, the spontaneous neoplasms of +/+ mice developed with a much lower frequency, and were more morphologically diverse than the gld tumors (65, 66). Consistent with the strain-related differences in tumor incidence observed in the present study, the incidence of B cell tumors in the BALB/c/+ mice was significantly higher than in the C3H/+/ mice (66). The persistence in strain differences in tumor susceptibility in the gld mice, suggests that the pro-oncogenic effects of the Fasl defect may complement, but not override, preexisting tumor susceptibility factors. The tumor accelerating effects of gld also are dependent on homozygosity as gld/+ mice showed no evidence of decreased lifespan or tumor development (data not shown). This contrasts with our earlier studies where we observed that SJL/lpr/+ mice developed B cell tumors more rapidly and died earlier than SJL/lpr/ mice (67). It has yet to be determined if this effect is lpr-dependent, strain-dependent, or both.

The loss of B220+ DN T cells with advanced age in lpr and gld mice has not been reported previously. The mechanisms leading to the depletion of these cells are unknown but may involve diminished production, or age-related exhaustion. Interestingly, CD8+ T cells, which are the putative progenitors of B220+ T cells (16–19), often were greatly increased in mice deficient in B220+ DN T cells. One explanation for this observation is that only a subset of CD8+ T cells can differentiate into B220+ DN T cells and that with time these either diminish in numbers or lose their capacity to differentiate. The loss of B220+ DN T cells and the development of B cell tumors appear to be coincidental events in the lpr and gld mice because many mice with greatly diminished numbers of B220+ DN T cells were tumor-free and not all tumor-bearing mice were depleted of DN T cells.

In conclusion, we have shown that inactivation of the Fas-mediated cell death pathway by mutation of Fas or Fasl accelerates the development and greatly increases the incidence of B cell lymphomas. It remains to be determined how the complex immunologic sequelae that result from these mutations contribute to the transformation process and whether they may be manipulated for therapeutic purposes.

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