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The Role of Environmental Antigens in the Spontaneous Development of Autoimmunity in MRL-lpr Mice1,2

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It has been proposed that the “normal” stimulation of the immune system that occurs from interactions with environmental stimuli, whether infectious or dietary, is necessary for the initiation and/or continuation of autoimmunity. We tested this hypothesis by deriving a group of MRL-lpr mice into a germfree (GF) environment. At 5 mo of age, no differences between GF and conventional MRL-lpr mice were noted in lymphoproliferation, flow cytometric analysis of lymph node cells (LN), or histologic analysis of the kidneys. Autoantibody levels were comparably elevated in both groups. A second experiment tested the role of residual environmental stimuli by contrasting GF mice fed either a low m.w., ultrafiltered Ag-free (GF-AF) diet or an autoclaved natural ingredient diet (GF-NI). At 4 mo of age, both groups showed extensive lymphoproliferation and aberrant T cell formation, although the GF-AF mice had ~50% smaller LNs compared with sex-matched GF-NI controls. Autoantibody formation was present in both groups. Histologic analysis of the kidneys revealed that GF-AF mice had much lower levels of nephritis, while immunofluorescence analysis demonstrated no difference in Ig deposits but did reveal a paucity of C3 deposition in the kidneys of GF-AF mice.

These data do not support a role for infectious agents in the induction of lymphoproliferation and B cell autoimmunity in MRL-lpr mice. Furthermore, they suggest that autoantibodies do not originate from B cells that were initially committed to exogenous Ags. They do suggest a possible contributory role for dietary exposure in the extent of lymphoproliferation and development of nephritis in this strain. The Journal of Immunology, 1999, 162: 6322–6330.

The role of environment in influencing systemic autoimmune disease is still an open issue. It is unknown to what extent the “normal” stimulation of the immune system that occurs through interaction with nonpathogenic environmental antigenic stimuli (microbiota and diet) influences the development of genetically programmed systemic autoimmunity. More specifically, the suggestion that the stimulation of B cells by exogenous Ags may influence the repertoire of autoantibodies provides a potential mechanism whereby ubiquitous environmental agents may play a role in the development of autoimmunity (1–3). Therefore, we proposed to evaluate the role of both normal microbiota and dietary Ags on the development of systemic autoimmunity in MRL-lpr mice.

Some previous studies have addressed the role of environmental stimuli, including the germfree (GF)1 state, in several models of autoimmunity in mice. Organ-specific autoimmunity was unaffected by GF conditions, as seen in the susceptibility to posttrectomy autoimmune gastritis in BALB/c mice (4). The transfer of myelin basic protein-specific TCR transgenic mice from a conventional to an specific pathogen-free (SPF) facility resulted in the suppression of the spontaneous development of experimental autoimmune encephalomyelitis (5). Previous work noted that New Zealand black (NZB) mice maintained under GF conditions had lower levels of serum IgG, although they had higher levels of antinuclear Abs (6, 7). Also noted were a lower incidence and later onset of moderate to severe renal disease (7). In contrast, transferring nonobese diabetic (NOD) mice from SPF to GF conditions resulted in a marked increase in the incidence of diabetes (8). These findings suggest that some features of systemic autoimmunity may be dependent on microbiota. They do not, however, address the possible role of dietary Ags, including bacterial byproducts.

In normal mouse strains, GF environment has a quantitative effect on the immune system. GF mice have smaller lymphoid tissue and reduced serum Ig levels. The GF state does not, however, abrogate their ability to respond to specific antigenic stimuli, although primary responses are often less vigorous than those of conventionally housed mice (9, 10).

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GF mice have never encountered live microorganisms, but they are routinely exposed to nonviable microbes, primarily in their diet (10). Alternatively, GF mice can be made “Ag-free” (AF), if they are fed an ultrafiltered, low m.w., chemically defined liquid diet and housed on ashless filter-paper bedding. Such GF-AF animals are as close to being immunologically shielded from nonself antigenic exposure as is currently possible (11, 12). The diet itself contains no potentially antigenic macromolecules, while ultrafiltration further guarantees the absence of intermediate m.w. Ags of bacterial origin, such as endotoxin, or viral contamination (10). The AF diet was originally developed for rigorous determination of nutritional requirements in GF mice and has been extensively characterized for its nutritional adequacy over several generations (11, 13–15).

Studies of the immunological parameters of GF-AF mice demonstrate a profound depression in levels of circulating IgG and IgA (with nearly normal IgM levels), low numbers of circulating white blood cells and reduced number of splenic Ig-secreting cells (10, 15, 16). In spite of these findings, GF-AF mice do have normal numbers of Ag-specific IgM-secreting cells, and the diversity of the IgM repertoire is conserved as measured by a quantitative immunoblot assay (16, 17). Primary responses to T cell-dependent Ag immunization actually produce higher numbers of Ag-specific IgG-secreting cells in GF-AF mice than in conventional controls (18). T cell-independent responses and affinity maturation following T cell-dependent immunizations are also comparable to what is seen in conventionally housed mice (19, 20). Analysis of LPS-induced B cell hybridomas demonstrated similar patterns of V\(_{\mu}\) gene family usage with a high frequency of reactivity to either self or bacterial Ags in both neonatal SPF and adult GF-AF mice, but not in adult SPF mice (21, 22). While GF-AF mice have lower numbers of activated T cells than do SPF mice, T cell-effector function, as measured in a variety of ways, is normal, as is the TCR V\(_{\beta}\) repertoire measured by flow cytometry analysis (19, 23–25). All this work suggests that background Ig synthesis is mostly driven by foreign antigenic exposure, but the basic ontogeny of the immune system is autochthonous.

In the present work, we investigated the role of environmental stimuli on the development of autoimmunity in MRL-lpr mice. These mice have the fas\(^{lpr}\) mutation, which affects the expression of Fas, a cell surface receptor member of the TNFR gene family, which is involved in the induction of apoptosis. They spontaneously develop an autoimmune syndrome characterized by elevated numbers of Ag-specific IgG-secreting cells, and the diversity of the IgM repertoire is conserved as measured by a quantitative immunoblot assay (16, 17). Primary responses to T cell-dependent Ag immunization actually produce higher numbers of Ag-specific IgG-secreting cells in GF-AF mice than in conventional controls (18). T cell-independent responses and affinity maturation following T cell-dependent immunizations are also comparable to what is seen in conventionally housed mice (19, 20). Analysis of LPS-induced B cell hybridomas demonstrated similar patterns of V\(_{\mu}\) gene family usage with a high frequency of reactivity to either self or bacterial Ags in both neonatal SPF and adult GF-AF mice, but not in adult SPF mice (21, 22). While GF-AF mice have lower numbers of activated T cells than do SPF mice, T cell-effector function, as measured in a variety of ways, is normal, as is the TCR V\(_{\beta}\) repertoire measured by flow cytometry analysis (19, 23–25). All this work suggests that background Ig synthesis is mostly driven by foreign antigenic exposure, but the basic ontogeny of the immune system is autochthonous.

Materials and Methods

Mice

MRL/MpJ-Fas\(^{lpr}\) (MRL-lpr) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained under conventional conditions at the University of North Carolina School of Medicine (Chapel Hill, NC) animal facility. Pregnant dams were sent as founders to the Gnotobiotic Division of the Medical School Animal Care at the University of Wisconsin-Madison (Madison, WI). A group of mice were rederived into GF housing via cesarean section from these pregnant dams and maintained under these conditions from then on (Table I). A second group of mice, the control group for experiment 1, was delivered in a conventional facility and maintained there for the duration of the experiment. Mice were fed a standard NI Purina brand mouse chow (no. 5010) and purified water ad libitum (all autoclaved for the GF mice). GF mice were periodically tested and determined to be free of parasites and aerobic and anaerobic microbial growth. Sentinel mice were also routinely tested and found to be negative for viral serologies. In experiment 2, a group of GF dams were placed on a chemically defined, low m.w., water soluble, ultrafiltered AF diet within 10 days of delivery and throughout nursing. The GF-AF offspring were maintained on this AF diet following weaning. The water soluble and lipophilic components of the diet were presented to the mice separately. The preparation and composition of the aqueous diet was as previously described (solution A in Ref. 27). The lipophilic component of the diet, consisting of the fat-soluble vitamins dissolved in chemically synthesized triglycerides, has been previously described (14). The diet did not contain polysorbates to avoid the known immunological effects and hepatotoxicity associated with such diets (11, 28). The techniques for sterilization of diets and equipment and for monitoring for microbial contamination was as described (14, 27). We are grateful to Fred Dickeson and Julian Pleasants (American Biogenetic Sciences, South Bend, IN) for their assistance with the ultrafiltration of the aqueous portion of the diet through a membrane with a m.w. cut-off of 10,000 Da. The GF-AF mice were maintained on shredded ashless filter-paper bedding. The paper bedding is consumed by the mice and is considered important for normal intestinal function in GF-AF mice (29). BALB/c mice used were obtained from the GF breeding colony maintained at the University of Wisconsin-Madison since 1973 and were housed under identical conditions as the MRL-lpr mice described above.

For both experiments, mice were bled at 3 mo of age and then again at the time of sacrifice. Mice from the first experiment were euthanized at 5 mos of age. The mice from the second experiment were euthanized at 4 mos of age, 6 days after the discovery of a monocontamination of the AF diet with a nonpathogenic Lactobacillus caseiffermes vs coryniformis (Microbial ID, Newark, DE) (30). Before autopsy, mice were weighed, scored for skin and ear lesions, and proteinuria assayed by dip stick using Uristix reagent strips (Miles Laboratories, Elkhart, IN). Spleens, all identifiable lymph node cells (LN), and both kidneys were removed for analysis.

Preparation of cells

Single-cell suspensions of all LN and spleens were made by passing them through cell strainers (Becton Dickinson, Franklin Lakes, NJ) in cold medium (RPMI 1640 with 15 mM HEPES, 5% FCS (HyClone, Logan, UT), 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin (Life Technologies, Gaithersburg, MD)). RBC were lysed with NH\(_4\)Cl, and cells were washed twice before counting by an automated cell counter (Coulter, Hialeah, FL).

Immunofluorescence staining and flow cytometry

In 96-well microtiter plates, 1.5 \(\times\) 10\(^5\) cells/well were stained in cold media containing 3% FCS and 0.1% NaN\(_3\). In general, first and second step
reagents were incubated with cells on ice for 30 min. Cell surface immunofluorescence analysis of LN and spleen cells was performed by two- and three-color flow cytometry analysis with size gating on the lymphocyte population. At least 10⁶ events were collected for each sample on a FACScan (Becton Dickinson Immunochemistry Systems, San Jose, CA) with Cytometry (Fort Collins, CO) or CellQuest (Becton Dickinson Immunochemistry Systems) data acquisition and software.

Reagents for immunofluorescence staining and flow cytometry
Reagents used for immunofluorescence staining included: anti-IgM (Bet-2-BNHS), anti-CD45R/B220-FTTC (RA3-6B2; PharMingen, San Diego, CA), anti-CD4 (172.4 overgrown supernatant; RM4-5-PE; PharMingen), anti-CD8 (31 M overgrown supernatant; 53-6.72-PE; PharMingen), anti-Thy1.2 (MtnT1 overgrown supernatant; 53-2-1-BNHS; PharMingen), SAv-Cy-Chrome (PharMingen), SAv-R-PE (Southern Biotechnology Associates, Birmingham, AL), and anti-rat κ light chain (MAR-18.5) (31). Two-color immunofluorescence was used to analyze TCR Vβ and TCR γδ expression separately in the CD4⁺, CD8⁺, CD4⁺CD8⁻ (analyzed by costaining with both fluoresceinated anti-CD4 plus CD8 Abs) T cell populations, as previously described (32).

Quantification of Ig and autoantibody levels by ELISA
In experiment 1, serum samples were assayed in duplicate for total IgG, IgG2a, and IgM levels, as well as the following autoantibodies: IgG anti-chicken chorbinin, anti-ssDNA, anti-P, and anti-Sm; and IgG3 rheumatoid factor (RF) anti-IgG2a activity (clone P1.17 [mouse IgG2a] as target and clone 2E6 F(ab')₂-BNHS (rat IgG1 anti-mouse IgG3) as the secondary reagent), as previously described (33–35). In Experiment 2, IgG anti-dsDNA and IgM RF anti-IgG2a were performed in addition (35, 36). Results for both male and female sera for each group were pooled, as no significant differences were noted between them. The Crithidia assay for detecting anti-dsDNA Ab was performed as previously described (37). Additional sera from non-SF MRL-lpr mice and nonautoimmune B6.C20 mice were also assayed in experiment 1 for comparison. In Experiment 9, sera from BALB/c mice housed under identical conditions as the MRL-lpr GF and GF-AF MRL-lpr mice were also assayed as nonautoimmune controls. Some results are reported in equivalent dilution factors (EDF) of standardized reference MRL-lpr sera, as previously defined by the formula: EDF = (dilution of standard reference sera, which gives the equivalent OD of the test serum) × 10⁻⁶ (38).

Kidney histopathology (light microscopy and immunofluorescence)
The presence and severity of nephritis was determined on hematoxylin-eosin light and immunofluorescence microscopy as previously described (37, 39). In Experiment 1, a blinded observer (Jennette) evaluated and graded the overall severity by light microscopy, while IgG, IgM, IgA, and C3 deposits were individually scored by immunofluorescence microscopy.

In experiment 2, a blinded observer (Madaio) evaluated and scored independently the severity of glomerular, interstitial, and vascular lesions by light microscopy. Similarly, the presence of glomerular, tubular basement membrane and vascular deposits of IgG and C3 by immunofluorescence microscopy was judged independently. Multiple sections at a minimum of two different levels were observed. Each section typically involved evaluation of >50 glomeruli, >25 blood vessels, and the interstitium contained within two to three longitudinal sections of kidney.

Statistics
Student’s t test was used to determine the statistical significance of differences between most groups. Fisher’s exact test was used for comparisons of Crithidia assay anti-dsDNA titers. Differences were considered significant at p < 0.05. The significance level for the results of the ELISAs was adjusted using the Bonferroni correction for multiple comparisons of means to a p < 0.0063 (0.05 ÷ 8 = 0.0063, where 8 equals the number of assays performed). Error bars in graphs represent the 95% confidence intervals (CI). The differences in kidney histological and C3 and IgG deposition scores were analyzed using the two-sample Wilcoxon rank-sum (Mann-Whitney) test.

Results

GF and GF-AF MRL-lpr mice
To determine the role of environmental stimulus on the development of spontaneous autoimmune disease in MRL-lpr mice, we performed two separate experiments with MRL-lpr mice (Table I). In experiment 1, we wanted to establish the role oflive, nonpathogenic stimuli. We housed one group of mice under conventional conditions, while a second group was reared in a GF state. In experiment 2, we wanted to isolate the mice from environmental antigenic stimuli as much as possible. For this experiment, two groups of GF mice were established. One group was fed a conventional, autoclaved NI (GF-NI) diet, while the second group was maintained on a chemically defined, low m.w., water soluble, ultrafiltered AF (GF-AF) diet.

At 5 mo of age, experiment I mice, reared in a GF environment, had body weights no different from those of conventional controls (Fig. 1A). In experiment 2, same sex comparisons demonstrated that differences in total body weight (TBW) were only present between GF-AF and GF-NI males at 4 mo of age (33 g vs 38 g, p < 0.01) (Fig. 2A). These differences may be due in part to differences in LN weights and bowel weight and content (GF-AF mice have a reduction in cecal size, as a percentage of body weight in experiment 1. A, TBW, spleen, and LN weights. B, Total LN cell and T cell subset counts by cell surface staining and flow cytometric analysis. Values are expressed as means ± 95% CI.
weight, from 10% to 3% compared with GF-NI mice and have very little luminal fecal content (12). Of note, 7/8 female, but only 1/9 male, GF-AF mice had ear lesions typical of MRL-1pr mice, while none of the GF-NI diet-fed mice had ear lesions at the time of sacrifice. None of the mice demonstrated clinical signs of inflammatory arthritis at the time of sacrifice.

Lymphoproliferation in GF and GF-AF MRL-1pr mice
Analyses of the LN and spleens collected at sacrifice are presented in Figs. 1 and 2. In experiment 1, the GF state did not affect the weight or total cell counts of either LN or spleens (Fig. 1, A and B), as both groups demonstrated the usual degree of lymphoproliferation seen in MRL-1pr mice, well in excess of the levels seen in normal C57BL/6 mice (31). LN T cell subset analysis likewise did not reveal any differences (Fig. 1B). There was also no significant difference in the percentage of γδ DN T cells between the groups (conventional, 1.6% vs GF, 2.5%; p > 0.05). Fig. 3 shows that T cell subset TCR Vβ-chain usage, as determined by flow cytometric analysis of LN cells, was indistinguishable between the groups of mice and included the selective expansion of V88.3+ DN T cells (32).

While all mice in experiment 2 had extensive lymphadenopathy, GF-AF males, but not females, had significantly lower total LN weights and cell counts than their GF-NI counterparts (Fig. 2, A and B). Cell surface marker expression analysis revealed that all mice demonstrated the characteristic expansion of lymphocyte subsets seen in MRL-1pr mice (Fig. 2B). No significant differences were found in the percentage of CD4+ T cells, CD8+ T cells, or B cells (B220+/sIgM+) in same sex comparisons. GF-AF mice tended to have lower absolute numbers of cells. There were small but significant differences in the percentage of DN T cells (data not shown) with larger differences in the total number of DN T cells (GF-AF vs GF-NI; female, 2123 vs 3360 and male, 821 vs 2014 × 10^6 cells; both comparisons p < 0.01).

Autoantibody production in GF and GF-AF MRL-1pr mice
All sera collected from mice at 3 mo of age and at the time of sacrifice were analyzed for levels of Ig isotypes and autoantibody specificities. At 3 mo of age, conventional and GF MRL-1pr mice in experiment 1 had roughly equivalent levels of all autoantibodies tested, although GF mice made higher levels of both total IgG and IgG2a (data not shown). At the time of sacrifice (5 mo), GF and conventional MRL-1pr mice, again, had similar levels of autoantibodies (Fig. 4). Conventional mice appeared to have higher levels of IgM and anti-ssDNA autoantibodies (p = 0.05 and 0.04, respectively; NS using the Bonferroni correction for multiple comparisons), but GF mice showed a trend toward higher titers in others (total IgG2a and anti-Sm). Anti-dsDNA was tested by the Crithidia assay when sufficient serum was available. Eight of nine 5-mo sera in the GF group (89%) had titers of at least 1/160, while only 6/14 mice in the conventional group (43%) showed a similar level of reactivity (p < 0.01).

Both groups of MRL-1pr mice in experiment 2, compared with either group of BALB/c mice, produced significantly higher titers of all serologies tested at 4 and 3 mo of age (Fig. 5, and data not shown). GF-AF MRL-1pr mice, compared with GF-NI MRL-1pr mice, however, trended toward lower titers of most serologies tested, a pattern also repeated in the BALB/c mice. While GF-AF MRL-1pr mice produced significantly lower titers of total IgG, anti-ssDNA, IgM, and IgG3 RF anti-IgG2a+ (Fig. 5, A, D, E, and G), they produced comparable titers of anti-Sm, IgM RF anti-IgG2a+, anti-chromatin, and anti-dsDNA (Fig. 5, B, C, F, and H) at 4 mo of age. Serologies at 3 mo of age, before the monoclonal antibody, demonstrated similar patterns (data not shown). It is of note that even in those assays in which GF-AF MRL-1pr mice produced lower average titers, there was significant overlap in the level of the individual titers between both groups of MRL-1pr mice.

Development of nephritis in GF and GF-AF MRL-1pr mice
Nephritis involving the glomeruli, interstitia and blood vessels with deposition of Ig and complement is a hallmark of the autoimmune syndrome of MRL-1pr mice. As seen in Fig. 6, A–C, GF MRL-1pr mice developed both light and immunofluorescence findings that were indistinguishable from those seen in conventional controls. (The patterns of IgM and IgA deposition were similar to those of IgG, data not shown). Both groups also demonstrated similar levels of proteinuria at the time of sacrifice (Fig. 7A).

Strikingly, GF-AF mice developed little glomerular disease by light microscopy (Fig. 6D; p < 0.05; similar results were seen for vascular and interstitial changes, data not shown). When the kidneys were analyzed by immunofluorescence, extensive and indistinguishable amounts of glomerular IgG deposits were present in both groups (Fig. 6E; p > 0.05; results for vascular and interstitial.

![FIGURE 2. TBW and lymphoproliferation of GF-AF (female n = 7, male n = 9) and GF-NI (female n = 11, male n = 9) MRL-1pr mice in experiment 2 at 4 mo of age. A, TBW, spleen, and LN weights. B, Total LN cell and T cell subset counts by cell surface staining and flow cytometric analysis. Values are expressed as means ± 95% CI.](http://www.jimmunol.org/Downloadedfrom)
Data shown are mean percentages. All SE are 1demonstrates no differences between the groups for: CD4 and DN T cells (Clpr play a role in the development of either the lymphoproliferation, Our results demonstrate that live, nonpathogenic microbiota do not

Discussion; F there where extensive deposits found in the control GF-NI mice that it was minimal in the kidneys of GF-AF mice. By contrast, the GF-AF group. Analysis of C3 deposition, however, revealed class deposition suggested a switch away from these subclasses in representative kidneys from each group for IgG2a and IgG3 sub-

The possibility that the changes resulting from the AF diet were due primarily to nutritional inadequacy is unlikely, although we cannot rule out that the particular constituents of the diet may have played a role. The AF diet was originally developed for rigorous determination of nutritional requirements and has been characterized as the most studied dietary model (14). AF diet-fed mice are usually smaller at weaning but as adults achieve weights comparable to those of NI diet-fed mice, in spite of their higher resting O2 consumption (40). Previous experiments in which diets deficient in any number of components led to the amelioration of autoimmune features, including nephritis, in MRL-lpr and NZB/W F1 mice were always associated with concomitantly significantly lower body weights (41, 42). Additional work in MRL-lpr females, however, showed that a nutritionally adequate diet low in fat, none of it saturated, compared with isocaloric, higher fat diets, can decrease the incidence and severity of proteinuria, autoantibodies, and mortality without significantly affecting body weights (43). Other forms of dietary interventions, such as calorlc restriction, fatty acid supplementation, or restriction of tyrosine and phenylalanine, are known to have some beneficial effects on the progress of nephritis in MRL-lpr or (NZB × NZW)F1 (NZB/W F1) mice (42, 44–50). The significant difference in the fat composition of our AF diet could thus account for the observed decrease in the renal pathology of AF mice (51). Similarly, NZB/W F1 mice fed a diet in which the protein source was replaced with synthetic amino acids (as in our AF diet) had delayed onset of their autoimmune disease, without abnormalities of body weight (50). In our experiments, the decreases in certain parameters of autoimmunity in the GF-AF group were probably not due to malnutrition, as the mice displayed no obvious differences in appearance or behavior and little difference in weight compared with their GF-NI controls. However, it is impossible to differentiate between a modest role for dietary Ags vs a “chemical” effect of the synthetic diet’s constituents.

One of the most striking features of the fay phenotype is the massive, nonmalignant lymphoproliferation that can be found in all strains of mice deficient in Fas or Fas ligand (FasL) expression. These expanded lymphocyte populations, particularly the aberrant DN T cells, may play a role in the development of the autoimmune features of the syndrome. Previous work by us and others has suggested that the DN T cells derive mostly from the postthymic CD8+ T cell population (31, 32, 52). This led to the suggestion that DN T cells arise because defects in the Fas-FasL signaling pathway prevent normal deletion of T cells that have encountered Ag in the periphery. The development of substantial numbers of DN T cells in our GF-AF mice supports the contention that the lymphoproliferation, including the DN T cells, does not predominantly arise from reactivity to foreign Ags, but rather is largely driven by recognition of self.

Our results offer some insight into the origin of autoantibodies in MRL-lpr mice. The GF-AF MRL-lpr mice produced elevated titers of all autoantibodies tested, although certain specificities were decreased compared with GF-NI MRL-lpr mice. Thus, dietary Ags play, at most, a modifying role on the autoimmune process. Significantly lower levels of serum IgG were found in all GF-AF mice and were not due to synthetic defects brought on by protein-calorie malnutrition as seen in other experimental models (53, 54). As previously mentioned, while background levels of deposits were comparable, data not shown). Staining of five to six representative kidneys from each group for IgG2a and IgG3 subclass deposition suggested a switch away from these subclasses in the GF-AF group. Analysis of C3 deposition, however, revealed that it was minimal in the kidneys of GF-AF mice. By contrast, there where extensive deposits found in the control GF-NI mice (Fig. 6F; p < 0.05). GF-AF mice from experiment 2 also demonstrated much less proteinuria than NI mice (Fig. 7B).

Discussion

Our results demonstrate that live, nonpathogenic microbiota do not play a role in the development of either the lymphoproliferation, autoantibody formation, or nephritis seen in MRL-lpr mice. It also confirms that TCR Vβ repertoire development is not dependent on interactions with infectious agents (25). The elimination of anti-
serum IgG are extremely low in GF-AF mice, as we found in our BALB/c controls (Fig. 5), GF-AF mice are capable of mounting high titer, specific Ab responses following Ag exposure. In addition, adult GF mice transferred from an NI diet to an AF diet demonstrate a dramatic fall of serum IgG levels after 3–4 mo (10). These findings imply that baseline IgG levels are predominantly driven by continued foreign antigenic stimuli. In contrast, then, the significant titers of autoantibodies produced by GF-AF MRL-1pr mice and the relatively high levels of their serum IgG must be driven by specific Ags, which can only be autologous. This is further supported by experiments that indicate that the production of autoantibodies in MRL-1pr mice is cognate and follows the

![Image of graphs A to H]

**FIGURE 4.** Serologies of GF and conventional MRL-1pr mice in experiment 1. Results of ELISAs from bleeds at 5 mo of age from GF MRL-1pr mice compared with MRL-1pr mice housed under conventional conditions (GF n = 9, diamonds; conventional n = 14, squares) showed comparable levels. Sera from B6.C20 (n = 3, triangles) and non-SPF MRL-1pr (n = 4, crosses) mice were also assayed for comparison in panels D–F, and H. X indicates geometric mean.

**FIGURE 5.** Serologies of GF-NI and GF-AF MRL-1pr mice in experiment 2. Results of ELISAs from bleeds at 4 mo of age demonstrate significant titers of autoantibodies in both groups, although GF-NI mice tended to have higher titers (GF-AF n = 16, diamonds; GF-NI n = 20, squares). Sera from nonautoimmune BALB/c mice of comparable ages reared under the same conditions were also assayed in parallel (GF-AF n = 7, triangles; GF-NI n = 11, crosses). X indicates geometric mean.
pattern of an Ag-driven response focused on the identified autoantigens, i.e., dsDNA, Ig, and Sm (55–59). Thus, the autoantibodies characteristic of MRL-\textit{lpr} mice do not originate from B cells that were initially committed to exogenous Ags, and their ongoing production must be driven by continued exposure to autoantigen. What remains undetermined is the nature and the site of the original self-antigenic stimuli. It is apparent, however, that foreign Ags neither initiate nor exacerbate (by epitope spreading, for example) the endogenous propensity to make autoantibodies.

Nonautoimmune prone strains of mice have been shown to develop spontaneously certain RF specificities, mostly IgA and IgM anti-IgG2\(\alpha\), that can vary depending on environmental factors (60). For example, DBA/2 mice housed under SPF conditions made RF, while animals kept under GF conditions in the same colony did not. Our present work showed parallel results, since GF-NI BALB/c mice made significant titers of IgM RF anti-IgG2\(\alpha\) (Fig. 5C), while the GF-AF BALB/c mice did not. On the other hand, the spontaneously autoimmune MRL-\textit{lpr} mice made high titers of RF, both IgM and IgG3, whether they were housed under conventional, GF, or GF-AF conditions (Figs. 4 and 5). Therefore, this autoantibody specificity can be driven by environmental stimuli in some situations, but can also be independent of foreign Ags.

It was striking to find that dietary Ags, but not microbiota, played a major role in the development of nephritis in MRL-\textit{lpr} mice. In the GF-AF group, paucity of C3 deposition in the setting of large amounts of IgG may point to an explanation of this observation. The development of nephritis may depend on the absorption of dietary Ags that participate as cofactors in the binding of complement by renal immune complexes. Alternatively, a switch in the pattern of Ig subclass deposited in the kidneys may account for the observed differences in the amount of complement deposited and the subsequent development of nephritis. Peng et al. (61) also reported a disassociation between renal Ig deposition and the development of nephritis. A change in the C3 binding capacity of IgG deposited in kidney lesions by GF-AF MRL-\textit{lpr} mice is possible in their model as well as ours. It is also possible that the absence of complement deposition is due to decreased serum levels due to the AF diet. This seems unlikely as decreased levels of serum complement in both humans and experimental animals have only been reported in the setting of severe protein-calorie malnutrition and inanition (62).

\textbf{FIGURE 6.} Kidney histopathology for experiment 1 (A–C) and experiment 2 (D–F). GF mice had similar findings to conventional mice in experiment 1. In experiment 2, GF-AF mice had milder histological scores and C3 deposition, in spite of similar deposits of IgG compared with GF-NI mice. Panels A and D show the histopathology score for light microscopy; panels B and E show the results of immunofluorescence for IgG; and panels C and F show C3 deposits. The differences in scores were only statistically significant for the results in panels D and F \((p < 0.05)\) by the Two-sample Wilcoxon rank-sum (Mann-Whitney) test. For experiment 1, nine GF and nine conventional mice were scored at 5 mo of age. For experiment 2, 16 AF and 20 NI were scored at 4 mo of age.
This work supports the view that the specific immunoregulatory mechanisms involved in the induction of tolerance to certain autoantigens, like those targeted in systemic lupus erythematosus (SLE), fail in MRL-\(lpr\) mice due to genetic features in T and B cells that are largely independent of foreign antigenic stimuli. The striking similarity between the profile of autoantibodies produced in MRL-\(lpr\) mice and humans with SLE suggests that the immunoregulatory mechanisms that fail in humans with SLE may also be primarily genetic.

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References


FIGURE 7. Proteinuria at time of sacrifice. While GF and conventional mice had similar levels of proteinuria in experiment 1, GF-AF mice had lower levels compared with GF-NI mice in experiment 2. Panel A shows the number of mice for each given range of proteinuria on a scale of 0–4+ for mice from experiment 1 (GF \(n = 9\); conventional \(n = 9\)) at 5 mo of age. Panel B shows the number of mice for each given range of proteinuria on a scale of 0–4+ for mice from experiment 2 (GF-AF \(n = 16\); GF-NI \(n = 20\)) at 4 mo of age.

Previous work in both MRL-\(lpr\) and NZB/W \(F_1\) mice has suggested that anti-retroviral gp70 Ag-Ab complexes (gp70 complexes) are important in the pathogenesis of renal disease (47, 65). As a low-calorie diet is able to decrease the level of gp70 complexes, it is possible that the decreased incidence of nephritis in our AF mice may be due to a decrease in gp70 complexes (66). This concern is less likely as Dutta et al. (67) first demonstrated that the expression of serum gp70 did not correlate with the levels circulating immune complexes nor the presence of nephritis in the progeny of NZB and SWR crosses. Andrews et al. (68) later demonstrated a lack of a direct relationship between serum gp70 complexes levels and the development of nephritis in MRL-\(lpr\) mice. Hence, nephritis, indistinguishable in severity and age of onset, can be found in mice with either high and low titers of gp70 or gp70 Ag-Ab complexes. Another autoantibody, IgG3 RF anti-IgG2a, has been implicated in the development of various pathological manifestations, including glomerulonephritis, seen in MRL-\(lpr\) mice (35). It is possible that the lower levels of this autoantibody in GF-AF mice may in part explain the decreased severity of glomerulonephritis.

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