Autoantibodies Produced Spontaneously by Young lpr Mice Carry Transforming Growth Factor β and Suppress Cytotoxic T Lymphocyte Responses

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Summary

Young MRL/MpJ-lpr (lpr) mice 8–12 wk old challenged with alloantigen had significantly lower specific cytolytic T lymphocyte (CTL) responses than control MRL/MpJ +/+ mice. Serum from lpr mice compared with serum from +/+ or normal C3H mice powerfully suppressed CTL responses in mixed lymphocyte cultures (MLC); absorbing lpr serum on protein G, adding antibody against transforming growth factor β (TGF-β) to cultures or dissociating immunoglobulin G (IgG) and TGF-β before additions to cultures prevented suppression. Apparently autoantibody, similar to IgG produced by normal mice in response to immunization, carries TGF-β which suppresses CTL responses in vivo and in vitro.

lpr mice spontaneously develop autoimmunity which is characterized by progressively severe splenomegaly, lymphadenopathy, hypergammaglobulinemia, and immune deficiency. lpr mice carry a mutation of the FAS gene (1, 2). How the absence of expression of the normal FAS gene product, which is involved in one pathway causing apoptosis, leads to autoimmunity is not understood; nevertheless, lpr mice are considered a useful model for studying some aspects of autoimmunity, e.g., systemic SLE, that occur in humans (3–7).

Normal mice immunized repeatedly with a single antigen develop high titers of specific antibody, and a small fraction of the specific IgG produced is associated with or carries TGF-β which suppresses CD8+ CTL responses to unrelated antigens (8, 9). We report here that IgG autoantibodies produced by lpr mice also have this property, and we suggest that such autoantibodies may play an important role in the pathogenesis of diseases caused by autoimmunity.

Materials and Methods

Mice, Sera, and Plasma. MRL/MpJ-lpr (lpr) and MRL/MpJ- +/+ (H2b) females 5–6 wk old were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c (H2d) and C3H/HeN mammary tumor virus-negative (H2k) females were purchased from the Frederick Cancer Research Production Facility (Frederick, MD). All mice were housed in the same barrier facility and fed sterile food and water. Most of the mice were small groups of three or four untreated, age-matched mice that became available to us at irregular intervals from another project. Anesthetized mice were exsanguinated by heart puncture. Sera were tested on the same day.

Immunizations. Mice under light anesthesia were injected in each hind foot pad with 0.05 μl of a suspension of either sheep erythrocytes (5%) or BALB/c spleen cells (2 × 10^6 cells/ml) suspended in RPMI-1640; injections were repeated every second day; mice were killed 4 and 6 d after the first injection for plaque forming cell (PFC) responses and 6 d after the first injection for CTL responses.

Cultures, Assays, and Reagents. Cell preparations, media, culture conditions, assays, and recording of data for CTL and PFC were the same as described in detail (8, 9). For most experiments each one-way MLC contained 5 × 10^5 normal C3H spleen cells as responders and 5 × 10^5 irradiated (2,000 rad) BALB/c spleen cells as stimulators in a final volume of 200 μl. In experiments comparing spleen cells from lpr mice with spleen cells from control mice as responders in one-way MLC, cultures were set up in 24- (macrocultures) as well as in 96-well plates (microcultures). Monoclonal murine antibody against murine TGF-β2, -β3 was lot B2332 from Genzyme Corp. (Cambridge, MA). 20 μl of antibody, 100 μg/ml, was added per culture of responder and stimulator cells 1 h before addition of murine serum to be tested.
Results

Young lpr Mice Have Usual Specific Antibody but Reduced Specific CTL Responses on Immunization with Exogenous Antigens. Six lpr and six + + mice 8–10 wk old were immunized in hind foot pads with SRBC. Two mice in each group were killed at 4 d and the remaining four mice at 6 d. At 4 d virtually all PFC (>90%) secreted IgM; 9,000 and 11,000 PFC/two lymph nodes from the two lpr mice, and 13,000 and 14,000 PFC for lymph nodes from the two + + mice; all spleens contained 2,000–4,000 PFC. By 6 d 80–90% of PFC secreted IgG; responses for individual lpr mice were 9,000, 52,000, 54,000, and 109,000 PFC/two lymph nodes, and 65,000, 72,000, 126,000, and 152,000 PFC/two lymph nodes for + + mice. Responses in spleens were low but comparable for the two groups. Though not controlled within the experiment, the magnitude and variability of responses for both groups were comparable to responses we have observed in many experiments using C3H mice immunized in the same way.

In contrast to B cell responses, CTL responses stimulated by immunization with allogeneic spleen cells were approximately 10-fold lower for lpr mice than for + + mice. For example, in one experiment four lpr mice and four + + mice 8 wks old were immunized in foot pads with allogeneic antigens. The lymph node cells from each mouse were assayed separately in triplicate. Pools were also prepared from lymph nodes and from spleens. The pools were assayed in triplicate against the sensitizing antigen, P815 target cells (H^k^) and also against EL-4 target cells (H^2^a) for nonspecific lysis. The results recorded in Fig. 1 are for popliteal lymph node cells assayed separately and for pooled spleen cells. The magnitude of difference between lpr and + + lymph nodes was almost identical while comparing the means for individual mice or the means of responses for pooled lymph node cells. All of the pools caused <10% lysis of EL-4 at E/T ratios of 100 or 50:1. The experiment was repeated using 17-wk-old mice with almost identical results except that spleens of lpr mice were larger (>3 × 10^8 cells/spleen compared with 1.5 × 10^8 cells/spleen for younger lpr mice).

Response of lpr and + + Spleen Cells in MLC. Spleen cells from each of three lpr mice (two 10 wk and one 16 wk old), three + + mice (10, 16, and 21 wk old), and two C3H mice (10 and 16 wk old) were cultured separately in MLC. In macrocultures, differences between CTL responses were less than twofold between any two of the eight mice; the means of responses for the three lpr mice at E/T ratios of 50:1, 12.5:1, and 3:1 were 84, 81, and 68% Cr release and for the three + + mice 85, 78, and 59% Cr release. Since the spleens of lpr mice were one and one half to two times larger than spleens of control mice, the numbers of reactive CTL must have been at least as high in spleens of lpr as in + + mice. Interestingly, however, CTL responses were lower for lpr mice than control mice when MLC were in microcultures. Microcultures have one tenth the volume and cell number as macrocultures, but cell density of sedmented cells in stationary cultures is approximately twofold lower because of the ratio of cell number to the area of the well bottom (10^7 cells/2.0 cm^2 versus 10^6 cells/0.32 cm^2). Whereas CTL responses were almost identical in macro- and microcultures for + + and C3H mice, responses were approximately fourfold lower for lpr cells in microcultures, possibly because the enlarged spleens of lpr mice contain increased numbers of CD4^-CD8^- or other cells which are neutral or indifferent but interfere with specific cell–cell interactions required for generating specific CTL. We have not tested this possibility directly, but we have observed the same difference between responses in macro and microculture when comparing spleen cells from normal C3H mice and C3H mice with splenomegaly caused by immunogenic tumors that have grown progressively for several weeks.

Sera or Plasma from lpr Mice Powerfully Suppresses CTL Responses in MLC. In four consecutive experiments, pools of fresh sera were prepared from groups, three each, of age-matched, untreated lpr, + + mice and in two of the experiments pools from two C3H mice. Pools were tested on the same day in MLC. None of the pools from + + or C3H mice caused suppression at a dilution of 1:800; in contrast, all pools obtained from lpr mice more than 6 wk old caused a greater than 4–10-fold suppression based on E/T ratios re-
required to cause comparable cytolysis of target cells (Table 1). In a fifth experiment, fresh serum pooled from three 9-wk-old lpr mice also caused >10-fold suppression at a dilution of 1:800, whereas pooled sera from age-matched ++ and C3H mice caused no suppression at this dilution (data not presented). Aliquots of these three pools were fractionated/concentrated and stored at −80°C. The three pools were tested again 3 mo later; lpr serum was again approximately 10 times more suppressive than the control sera whether estimated by comparing the E/T ratio required to cause equivalent cytolysis or estimated by comparing dilutions of serum causing equivalent suppression (Table 2).

These results using pooled sera were confirmed in a sixth experiment using fresh plasma from four lpr and four ++ 9-wk-old mice and assayed individually. The mean percent ⁵¹Cr-release for 12 control cultures with medium were 75, 54, and 26 at E/T ratios of 100:1, 25:1, and 6.2:1. All four plasma samples from lpr mice abolished CTL responses at dilutions of 1:600 and 1:1800. All four plasma samples from ++ mice caused a less than twofold or no suppression at a dilution of 1:1800; at a dilution of 1:600, the lowest response at E/T ratios of 100:1, 25:1, and 6.2:1 was 32, 27, and 7%, and the mean responses for the group were 50, 34, and 10% at these E/T ratios.

**Table 1.** Sera from lpr Mice Suppress CTL Responses in MLC

| Exp | Medium or serum | Age of mice wk | 50:1 E/T ratio | 12.5:1 | 3:1 |
|-----|----------------|----------------|---------------|--------|-----|
| 1   | Medium control, 6 wk | – | 94 ± 10 | 61 ± 23 | 25 ± 10 |
| 2   | Medium control, 8 wk | – | 84 ± 8 | 57 ± 13 | 23 ± 7 |
| 3   | Medium control, 11 wk | – | 89 ± 6 | 62 ± 8 | 24 ± 5 |
| 4   | Medium control, 17 wk | – | 87 ± 5 | 73 ± 10 | 43 ± 14 |
| 1   | lpr | 6 | 75 | 28 | 25 |
| 2   | lpr | 8 | 48 | 19 | 8 |
| 3   | lpr | 11 | 21 | 7 | 3 |
| 4   | lpr | 17 | 53 | 17 | 3 |
| 1   | ++ | 6 | 100 | 63 | 25 |
| 2   | ++ | 8 | 96 | 59 | 22 |
| 3   | ++ | 11 | 87 | 67 | 34 |
| 4   | ++ | 17 | 91 | 67 | 30 |
| 3   | C3H | 11 | 97 | 77 | 35 |
| 4   | C3H | 17 | 100 | 87 | 39 |

Results are for four consecutive experiments; each serum was a pool from three lpr, three ++, or two C3H mice of the same age. All sera were diluted 1:800. The numbers recorded are means ± SD for 10 or more replicate cultures for medium and for duplicate cultures for each pool of serum.

**Table 2.** Fractionation and Concentration of Serum from lpr Mice

| Exp | Medium or serum | Age of mice wk | 50:1 E/T ratio | 12.5:1 | 3:1 |
|-----|----------------|----------------|---------------|--------|-----|
| 1   | Medium control, 6 wk | – | 94 ± 10 | 61 ± 23 | 25 ± 10 |
| 2   | Medium control, 8 wk | – | 84 ± 8 | 57 ± 13 | 23 ± 7 |
| 3   | Medium control, 11 wk | – | 89 ± 6 | 62 ± 8 | 24 ± 5 |
| 4   | Medium control, 17 wk | – | 87 ± 5 | 73 ± 10 | 43 ± 14 |
| 1   | lpr | 6 | 75 | 28 | 25 |
| 2   | lpr | 8 | 48 | 19 | 8 |
| 3   | lpr | 11 | 21 | 7 | 3 |
| 4   | lpr | 17 | 53 | 17 | 3 |
| 1   | ++ | 6 | 100 | 63 | 25 |
| 2   | ++ | 8 | 96 | 59 | 22 |
| 3   | ++ | 11 | 87 | 67 | 34 |
| 4   | ++ | 17 | 91 | 67 | 30 |
| 3   | C3H | 11 | 97 | 77 | 35 |
| 4   | C3H | 17 | 100 | 87 | 39 |

Results are for four consecutive experiments; each serum was a pool from three lpr, three ++, or two C3H mice of the same age. All sera were diluted 1:800. The numbers recorded are means ± SD for 10 or more replicate cultures for medium and for duplicate cultures for each pool of serum.
Table 2. Suppression of CTL Responses by Serum from 9-wk-old lpr Mice

| Medium or serum | Serum dilution | 50:1 | E/T ratio 12.5:1 | 3:1 |
|-----------------|----------------|------|-----------------|-----|
| Medium          | -              | 87 ± 7 | 65 ± 1         | 33 ± 10 |
| lpr             | 1:300          | 22 ± 6 | 0              | 0    |
|                 | 1:900          | 7 ± 5  | 0              | 0    |
|                 | 1:2700         | 44 ± 31 | 24 ± 31    | 6 ± 11 |
| ++              | 1:300          | 57 ± 24 | 27 ± 17     | 8 ± 6  |
|                 | 1:900          | 89 ± 8  | 72 ± 13       | 31 ± 10 |
|                 | 1:2700         | 92 ± 5  | 71 ± 7        | 28 ± 4  |
| C3H             | 1:300          | 90 ± 4  | 70 ± 10       | 30 ± 8  |
|                 | 1:900          | 87 ± 6  | 70 ± 5        | 28 ± 7  |
|                 | 1:2700         | 81 ± 4  | 63 ± 5        | 22 ± 3  |

Sera were pools from three lpr, three ++, and two C3H mice; results are for >100-kD fractions reconstituted to equal the volume before fractionation. The numbers recorded are means ± SD for 18 replicate cultures for medium and 3 replicate cultures for each serum dilution.

Figure 2. Suppression of CTL responses in MLC caused by lpr serum is removed by protein G (A) and prevented by antibody (AB) to TGF-β (B). The results are the means ± SD for 10 replicate control cultures for medium alone and for each of the variables tested.
but generalized lymphadenopathy is absent and total Ig levels are only slightly elevated (3, 4). Both lpr and repeatedly immunized mice have usual antibody responses but reduced CTL responses to appropriate immunization. High dilutions of sera or plasma from these mice suppress CTL responses in culture, and removal of IgG by absorbing on protein G or adding antibody against TGF-β to cultures prevents suppression. Furthermore, serum from either lpr or immunized mice which has been treated by acidification to dissociate TGF-β from IgG no longer causes suppression.

The antigen specificity of antibody obtained from immunized normal mice is irrelevant for causing suppression of CTL responses. (We have tested immunizing with allogeneic cells from three mouse strains, cells from three cell lines derived from different syngeneic immunogenic tumors, xeno-geneic erythrocytes from two species and TNP-KLH.) In fact, for selected experiments, we have purposely immunized normal mice with multiple antigens in order to boost the capacity of sera to suppress or to increase recovery of IgG-TGF-β from eluates or supranatants from cultured immunized cells. Autoantibodies in lpr mice are apparently the product of polyclonally stimulated B cells (10, 11) which have specificities for epitopes on ribonucleoproteins, histones, and DNA (12–15); the predominant isotype of these autoantibodies is IgG2a (16, 17). 2–3-mo-old lpr mice have only slightly elevated serum IgG levels and probably do not have circulating antigen–antibody complexes which appear later and cause complications such as nephritis in older lpr mice (6, 7).

Collectively, the findings suggest that IgG autoantibodies, presumably of multiple specificities, and IgG antibodies produced in response to specific immunization are comparable for suppressing CTL responses, and in both cases suppression is mediated by TGF-β carried by IgG. Macrophages and functional Fc receptors are obligatory for IgG-TGF-β to cause suppression (9). We have recently demonstrated that isolated single B cells secrete IgG-TGF-β with TGF-β in the latent form and that antigen–antibody complexes are not required and may interfere with suppression (our manuscript in preparation). Presumably IgG as a binding protein for the secretion of latent TGF-β focuses through Fc receptors the local activation of TGF-β by macrophages. CD8+ CTL are essential for effective host resistance to many, possibly all, viral infections (18–20). We proposed that IgG-TGF-β produced in response to a growing tumor or some viral infections might promote escape of tumor or viral variants that are observed to emerge during tumor growth or infection (8). We now suggest that production of autoantibodies during the early stages of diseases such as SLE may suppress the capacity of the individual to respond effectively to first or new viral infections. For this reason viral infections which are relatively innocuous for normal individuals may complicate the pathogenesis of autoimmune. It is also conceivable that TGF-β carried by IgG and focally activated where autoantibodies combine with autoantigens may stimulate connective tissue proliferation and scarring, characteristics of many autoimmune diseases.
References

1. Watanabe-Fukunaga, R., C.I. Brennan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature (Lond.). 356:314–317.

2. Wu, J., T. Zhou, J. He, and J.D. Mountz. 1993. Autoimmune disease in mice due to integration of an endogenous retrovirus in an apoptosis gene. J. Exp. Med. 178:461–468.

3. Andrews, B.S., R.A. Eisenberg, A.N. Theofilopoulos, S. Izui, C.B. Wilson, P.J. McConahey, E.D. Murphy, J.B. Roths, and F.J. Dixon. 1978. Spontaneous murine lupus-like syndromes. J. Exp. Med. 148:1198–1215.

4. Pisetsky, D.S., G.A. McCarty, and D.V. Peters. 1980. Mechanisms of autoantibody production in autoimmune MRL mice. J. Exp. Med. 152:1302–1310.

5. Rathmell, J.C., and C.C. Goodnow. 1994. Effects of the lpr mutation on elimination and inactivation of self-reactive B cells. J. Immunol. 153:2831–2842.

6. Jevnikar, A.M., M.J. Grusby, and L.H. Glimcher. 1994. Prevention of nephritis in major histocompatibility complex class II-deficient MRL-lpr mice. J. Exp. Med. 179:1137–1143.

7. Kim, C., K.A. Siminovitch, and A. Ochi. 1991. Reduction of lupus nephritis in MRL-lpr mice by a bacterial superantigen treatment. J. Immunol. 148:1561–1569.

8. Weidt, G., O. Utermöhlen, J. Zerrahn, J. Reimann, W. Depert, and F. Lehmann-Grube. 1994. CD8+ T lymphocyte-mediated antiviral immunity in mice as a result of injection of recombinant viral proteins. J. Immunol. 153:2554–2561.

9. Bertoletti, A., A. Costanzo, F.V. Chisari, M. Levereto, M. Artini, A. Sette, A. Penna, T. Giuberti, F. Fiaccadori, and C. Ferrari. 1994. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. J. Exp. Med. 180:933–943.

10. Koup, R.A. 1994. Virus escape from CTL recognition. J. Exp. Med. 180:779–782.