Brief Definitive Report

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-+ (++) ($H2^k$) females 5–6 wk old were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c ($H2^d$) and C3H/HeN mammary tumor virus–negative ($H2^k$) 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. Aliquots were diluted 1:10 in RPMI-1640 and concentrated into >100-kD fractions by filtration/centrifugation using Centricon microconcentrators (Amicon, Beverly, MA). The procedure reduced original volumes by 1.5–3-fold, and samples were stored at −80°C until used. The original volumes of fractionated sera were restored before dilution; dilutions of sera recorded are final concentrations in culture. Plasma was prepared from individual mice by diluting 50 μl of fresh blood obtained from the orbital plexus in 950 μl complete medium; samples were centrifuged and the cell free supranatant was considered diluted 1:20. Sera were absorbed on protein G using HiTrap™G (Pharmacia LKB Biotechnology, Uppsala, Sweden). Sera were diluted in a 0.1 M $NaPO_4$ "binding buffer;" 1.0 ml aliquots was either untreated or added to columns (∼2.0 min for additions and 10 min for absorption, the procedure repeated twice). Samples were diluted in complete media for testing in one-way MLC. Concentrations of IgG were determined in a modified ELISA assay using goat anti-murine IgG (H + L) conjugated to alkaline phosphatase (Calbiochem-Novabiochem Corp., La Jolla, CA).

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 (2×10^8 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). Cell number and volume were 10-fold higher in macrocultures. 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.

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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 alloantigen. 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^{2k}) and also against EL-4 target cells ($H2^{b}$) 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% ^{51}Cr release and for the three + + mice 85, 78, and 59% ^{51}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 sedimented 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.

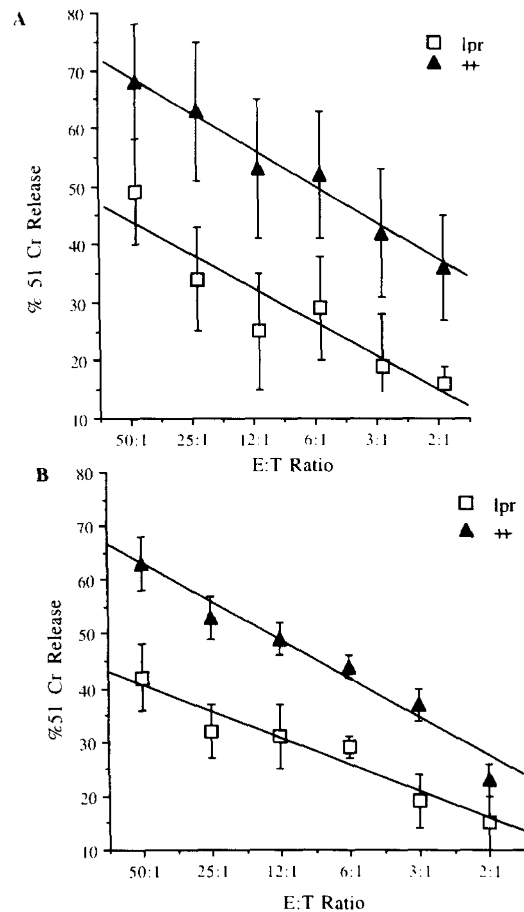

Figure 1. Young *lpr* mice have lower CTL responses on sensitization with alloantigen than control + + mice. (*A*) Popliteal lymph nodes assayed individually in triplicate; (*B*) Spleens pooled and assayed in triplicate. Results are the means for four mice per group ± SD.

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-

quired 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 ^{51}Cr-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.

Suppression Caused by lpr Serum Is Removed by Absorption on protein G or Is Prevented by Antibody to TGF-β. Aliquots of the same sera used in the experiment described in Table 2 (fractionated/concentrated, stored, and reconstituted) from 9-wk-old mice were either untreated or absorbed on protein G. The unbound and absorbed samples were then diluted in culture medium and added to MLC. Untreated *lpr* serum caused >4-fold suppression at a dilution of 1:1200, data not shown, and >10-fold suppression at a dilution of 1:400. Absorption of *lpr* serum on protein G greatly reduced suppression caused by serum diluted 1:400 (Fig. 2 *A*). The control serum absorbed on protein G had no effect on CTL responses. Aliquots of the same sera from 9-wk-old mice were used in a separate experiment for testing the effect of anti-TGF-β antibody on suppression. In the experiment shown in Fig. 2 *B*, *lpr* serum diluted 1:600 or 1:1200 abolished CTL responses. The anti-TGF-β antibody at the concentration used increased responses suppressed by *lpr* serum diluted 1:600 by approximately fourfold (data not shown); anti-TGF-β antibody restored responses suppressed by *lpr* serum diluted 1:1200 (Fig. 2 *B*) and did not affect responses to control serum. In other experiments, murine IgG2a of unknown specificity used at the same concentration as the anti-TGF-β had no effect on CTL responses suppressed by immune sera. The results reported in Fig. 2, *A* and *B* were confirmed using different pooled *lpr* and + + sera (data not shown).

Dissociation of IgG and TGF-β Eliminates Suppression Caused by lpr Serum. IgG from murine sera contains 1.0–10.0 ng

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

Exp	Medium or serum	Age of mice	Percent ^{51}Cr release, E/T ratio 50:1	12.5:1	3:1
		wk			
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	Percent ^{51}Cr release, E/T ratio 50:1	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.

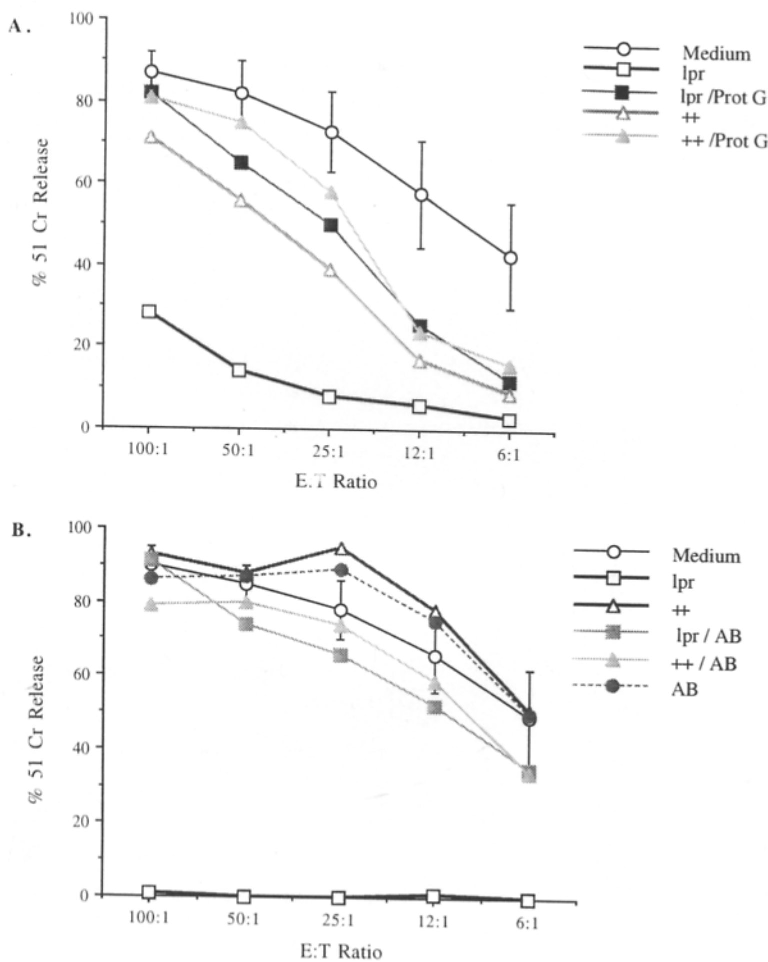

Figure 2. Suppression of CTL responses in MLC caused by *lpr* serum is removed by protein G (*A*) and prevented by antibody (*AB*) to TGFβ (*B*). The results are the means ± SD for 10 replicate control cultures for medium alone and for each of the variables tested.

latent TGF-β/mg IgG; acidification of immune sera or purified IgG, pH 2.0, for 30 min, cleaved active TGF-β from the latent form carried by IgG (9). Acid-treated serum, though retaining full antibody activity and containing TGF-β biologically active in a bioassay, no longer suppressed CTL responses in high dilution, presumably because IgG no longer focused through Fc receptors precise localization/activation of the latent TGF-β carried by IgG (9). Similarly acidification of *lpr* serum greatly reduced the capacity of *lpr* sera to suppress; for example in one experiment serum from *lpr* and ++ mice were diluted 1:25 in normal saline; one aliquot was acidified to pH 2.0 with 1.2 N HCl and neutralized with 0.72 N NaOH 30 min later; the same quantities of acid and base were added simultaneously to control aliquots at the beginning of the procedure. At the end of 30 min the samples were diluted in medium and added to MLC. The means for ^{51}Cr release of 20 control cultures with only medium or saline were 90, 82, and 49% at E/T ratios of 50:1, 12.5:1, and 3:1. Sera from ++ mice, treated or untreated at a dilution of 1:400 had little or no effect on cultures. Sera from *lpr* mice treated simultaneously with acid and base abolished responses at a dilution of 1:400 (<10% ^{51}Cr release at all E/T ratios) and acidification for 30 min greatly reduced this suppression (70, 35, and 12% ^{51}Cr release at E/T ratios of 50:1, 12.5:1, and 3:1).

Discussion

Young 8–12-wk-old *lpr* mice are in many ways comparable to normal mice immunized every 2 or 3 d for several weeks. The mice appear vigorous and healthy; spleens are enlarged 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, xenogeneic 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 autoimmunity. 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.

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