INDUCTION OF MURINE AUTOIMMUNE DISEASE BY CHRONIC POLYCLONAL B CELL ACTIVATION*

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The most distinguishing and earliest common immunological abnormality shared by all three murine models of accelerated systemic lupus erythematosus (SLE),[1] MRL/l, BXSB males, and NZB × NZW females is B cell hyperactivity (1, 2). These hyperactive B lymphocytes express (*a*) early spontaneous polyclonal activation, (*b*) increased frequency of colony formation and Ig secretion, and (*c*) excess autoantibody production. These features are present early in the mice with accelerated SLE but not in their late life SLE-prone counterparts (female MRL/n, BXSB, NZW) or in immunologically normal murine strains (BALB/c and C57BL/6). The underlying mechanisms responsible for this early, generalized B cell activation and autoantibody production are not known but presumably could involve intrinsic B cell abnormalities, regulatory cell defects, or excessive actions of one or more endogenous or exogenous B cell stimulators.

Lipopolysaccharide (LPS), derived from gram-negative bacteria, is a potent in vivo and in vitro stimulator of B lymphocytes and may be considered a prototype of exogenous predominantly T-independent polyclonal B cell activator (PBA) (3). In the absence of any specific antigenic influence, LPS can trigger proliferation and differentiation of B lymphocytes with production of Ig and autoantibodies. A single injection of LPS into normal mice will lead to transient formation of autoantigen-antibody immune complexes both within the circulation and also as deposits in glomeruli but without the development of histologically detectable glomerulonephritis (GN) (4, 5). Furthermore, the lipid A portion of LPS, which is mitogenic and minimally antigenic (6), can induce long-lasting production of both IgM and IgG autoantibodies (6–8).

The purpose of our present study was to examine the pathogenic potential of such exogenously-induced autoimmunity by asking the questions: (*a*) Can chronic B cell activation alone induce significant autoimmune disease (AID)? and (*b*) What role(s), if any, might a genetically-determined host predisposition play in the development of such disease? We found that chronic stimulation with the nonantigenic but mitogenic

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[1] *Abbreviations used in this paper:* AID, autoimmune disease; BUN, blood urea nitrogen; CIC, circulating immune complex; DVD, degenerative vascular disease; FDP, fibrinogen degradation products; FITC, fluorescein isothiocyanate conjugated; GN, glomerulonephritis; LPS, lipopolysaccharide; PAS, periodic acid-Schiff; PBA, polyclonal B cell activator; PFC, plaque-forming cells; SLE, systemic lupus erythematosus; ssDNA, single-strand DNA.

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lipid A fraction of LPS, beginning early in life, can greatly accelerate the onset of late-life SLE disease of MRL/n, BXSB, and NZW females, as evidenced by the early increase of Ig-secreting splenocytes, hypergammaglobulinemia, autoantibody production, and fatal immune complex-mediated GN. Similar chronic mitogen stimulation had less effect in immunologically normal mice which developed a much milder form of AID and no effect in LPS nonresponder C3H/HeJ mice.

Materials and Methods

Mice. All mice used in this study were 6–10 wk old. Late-life SLE-prone mice (female MRL/n, BXSB, and NZW), normal mice (BALB/c and C57BL/6 females), and LPS nonresponder mice C3H/HeJ (9) were all obtained from the murine breeding colony of the Scripps Clinic and Research Foundation.

Lipid A-LPS (R595). Lipid A-LPS purified from *Salmonella minnesota* was purchased from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA. The R595 preparations were diluted to 125 μg/ml with saline for injection. All six groups of mice were given 25 μg R595 in a 0.2 ml vol i.p. twice a week beginning at 6 wk of age.

Heparin. Heparin Sodium Injectable, purchased from Invenes (Chagrin Falls, OH) was used for anticoagulation to rule out possible thrombotic complications (Schwartzman reaction) induced by the LPS endotoxin, which might contribute to tissue injury. Additional representative groups of mice, both late-life, SLE-prone (MRL/n), and non-SLE (BALB/c, C3H/HeJ) strains received 1 U of heparin/g body weight, subcutaneously, 30 min before each R595 injection. The effectiveness of heparinization was tested by measuring the whole blood clotting time of the treated mice sampled at 30 min after R595 injections.

Serological assays. Anti-single-strand DNA (ssDNA) antibody activity was determined by a modified Farr DNA-binding radioimmunoassay (10). Circulating immune complexes (CIC) in individual sera were quantitated by two different assays: (*a*) a modified Raji cell radioimmunoassay using aggregated mouse gammaglobulin (AMG) to prepare the standard curve (11), and (*b*) Ig-bound retroviral envelope gp70 complexes, expressed as micrograms of gp70 bound to Ig detected by absorption of sera with staphylococcal protein A (12) (Calbiochem-Behring Corp.). Serum levels of IgM, IgG1, IgG2a, and IgG2b were determined by radial immunodiffusion (13) in commercially prepared agar plates (Meloy Laboratories Inc., Springfield, VA) containing rabbit anti-mouse isotype and subclass specific antisera; IgG3 levels were similarly assessed using monospecific rabbit-anti-mouse antisera (Litton Bionetics, Kensington, MD). Blood urea nitrogen (BUN) levels were semiquantitatively measured with Azostix (Ames Div., Miles Laboratory Inc., Elkhart, IN) with values ranging from 0.1 to 0.6 mg/ml. BUN levels of >0.3 mg/ml were considered as indications of compromised renal function.

Splenic Lymphocyte Plaque Assay. Spleen cells secreting IgG1, IgG2a, IgG2b, IgG3, and IgM were detected with subclass-specific facilitating antisera used in a reverse plaque assay using protein A-coupled sheep erythrocytes (SRBC) (14). Pooled spleen cells of four to six mice from each of the six R595-treated groups and their corresponding unmanipulated controls were tested after 2 and 4 mo of R595 treatment for quantitation of plaque-forming cells (PFC). The facilitating antibodies used in this study were purchased from Gateway Immunosera, Cahoka, IL, and rendered subclass specific by solid-phase immunoadsorption. Specificity and efficiencies of the facilitating antisera were verified using a panel of mouse plasmacytomas and/or hybridomas secreting antibodies of the major mouse Ig isotypes. Facilitating antisera were diluted ~1:2,000 to give near optimal plaquing efficiencies.

Histopathology. Representative mice from each group were autopsied at bimonthly intervals and sections of major organs were processed and stained with PAS for histological examination. GN was quantitated on a 1 to 4+ scale based on the severity and extent of histopathological changes. A grade 1 lesion showed minimal mesangial thickening, 2+ lesions contained noticeable increases in both mesangium and in glomerular cellularity, 3+ lesions were characterized by the preceeding features plus superimposed inflammatory exudates and/or capsular adhesions, and 4+ lesions had obliteration of glomerular architecture involving ≥70% glomeruli. Grade 3–4+ GN were considered significant contributors to cause of death resulting from autoimmune disease.

Other histological manifestations of autoimmune disease were also evaluated and documented. They included (*a*) lymphoproliferation, measured by the total increased area of splenic white pulp as compared with unmanipulated controls; (*b*) degenerative vascular disease (DVD), defined as presence of PAS^+ deposits along and within coronary vessel walls and lumens; and (*c*) arteritis, identified by presence of necrotizing and exudative inflammation involving the walls of small to medium sized arteries.

Detection of Ig and Fibrinogen Deposits in Renal Glomeruli. One-half of each kidney obtained from mice of the various groups at autopsy was frozen in liquid nitrogen and stored at −70°C. Frozen sections were cut and processed for staining with fluorescein isothiocyanate (FITC) rabbit-anti-mouse IgG and goat anti-mouse fibrinogen (N. L. Cappel Laboratories, Cochranville, PA). The intensities of fluorescence within renal glomeruli were graded from 0 to 4+.

Detection of Fibrinogen Degradation Products (FDP). To identify possible endotoxin-induced coagulopathy as a result of the repeated R595 injections, plasma from individual mice at 1 h pre- and 1, 2, and 24 h post-R595 injections were obtained and analyzed for presence of FDP. FDP were detected by immunoelectrophoresis based on their enhanced mobility relative to intact fibrinogen (15). Mouse fibrinogen was isolated from plasma by differential ethanol precipitation (16) and antiserum to fibrinogen was raised in rabbits. The antiserum was rendered specific for fibrinogen by absorption with freshly prepared mouse serum (1 part mouse serum to 50 parts rabbit antiserum). Immunoelectrophoresis was performed in 1% agarose (SeaKem ME; Marine Colloids, Rockville, MD) in 0.03 M sodium barbital, pH 8.3 on 2 × 3-inch glass slides. 5 μl plasma samples were applied and electrophoresed at 150 V for 8 h. Troughs were then removed, 50 μl antiserum was added, and precipitin lines were developed at 4°C for 24 h. Based on positive controls developed by the addition of plasmin to isolated mouse fibrinogen or mouse plasma, the experimental samples were recorded as plus or minus. The sensitivity of the system was estimated to be ~50 μg FDP/ml.

Results

Induction of Autoantibody Production by R595. Table I displays the various serological parameters of the six different groups of mice at 5 mo of age, after 3 mo of chronic stimulation with R595. As compared with the unmanipulated controls, all treated groups except the C3H/HeJ had hypergammaglobulinemia and increased anti-ssDNA antibodies. Similarly, CIC, measured by the Ig-bound retroviral gp70 and

TABLE I

Serological Parameter of R595-treated Mice and Unmanipulated Controls (5 Mo of Age)

	R595 treatment	Total Ig	Percent binding anti-ssDNA	Ig bound gp70	Raji cell assay
		mg/ml		*μg/ml*	*μg AMG/ml*
MRL/n ♀	+	19	50	22.8	70
	−	5	34	1.6	12
BXSB ♀	+	18	63	17.0	89
	−	4	15	1.0	5
NZW ♀	+	15	46	11.1	37
	−	4	15	3.0	4
BALB/c♀	+	22	48	1.4	22
	−	5	10	0.2	<1
C57BL/6♀	+	18	34	1.8	25
	−	3	4	0.3	<1
C3H/HeJ♀	+	4	6	0.4	<1
	−	4	5	0.2	<1

TABLE II

IgM and IgG Subclass Responses (PFC) in R595-treated Mice and Unmanipulated Controls (4 Mo of Age) PFC × 10^3/Spleen

	R595 treatment	IgG3		IgG1		IgG2a		IgG2b		IgM	
MRL/n	+	466	(33)*	37	(9)	50	(8)	98	(9)	2369	(30)
	−	14		4		6		11		80	
BXSB ♀	+	486	(120)	37	(5)	39	(5)	525	(58)	1700	(13)
	−	4		8		8		9		130	
NZW♀	+	189	(19)	77	(5)	138	(34)	60	(12)	3625	(4)
	−	10		17		4		5		1032	
BALB/c♀	+	500	(83)	85	(12)	80	(8)	87	(8)	6875	(46)
	−	6		7		10		10		150	
C57BL/6♀	+	496	(82)	43	(5)	44	(10)	258	(14)	8125	(67)
	−	6		5		4		18		125	
C3H/HeJ♀	+	5	(1)	4	(1)	7	(1)	10	(1)	200	(1)
	−	.5		4		6.5		12		180	

* Measured as stimulation index (treated/untreated control) of PFC/10^6 spleen cells.

Raji cell assay, were increased significantly in all groups except C3H/HeJ; the absolute amounts of CIC, however, were less in the treated normal groups than in the treated late-life SLE mice.

Polyclonal B Cell Activation by R595. The extent of polyclonal B cell activation by R595 was assayed by measuring the numbers of splenic lymphocytes secreting IgG of the different subclasses and IgM. Table II lists the PFC results obtained from the treated and control groups 2 mo after initiation of stimulation (4 mo of age). All treated mice, except C3H/HeJ, showed varying increases in numbers of splenic lymphocytes secreting each of the four IgG subclasses and IgM (as reflected by the calculated stimulation indices). The patterns of Ig enhancement differed among the individual strains, i.e., BXSB and C57BL/6 females had marked increases in IgG3 (120 and 82 times, respectively) and IgG2b (58 and 14 times); MRL/n and BALB/c females had greater increases in IgG3 (33 and 83 times, respectively) than any of the other IgG subclasses; and NZW mice had most enhancement of IgG3 and IgG2a. The serum levels of IgG subclasses, measured at 5 mo of age, also indicated selective subclass enhancement in all but the C3H/HeJ mice, with greatest increases in IgG3 and IgG2b (Table III).

Induction of Glomerulonephritis with R595. After 2 mo of R595 injections, all five groups of mice (except C3H/HeJ) showed grade 2+ GN with BUN levels ranging from 0.1 to 0.3 mg/μl (Fig. 1). After prolonged stimulation for 6–8 mo, the late-life SLE-prone mice had progression of their GN, i.e., grade 4+ and BUN of 0.6 mg/μl, whereas normal mice showed no significant progression beyond that seen after 2 mo. None of the unmanipulated controls, at 10 mo of age, had significant BUN elevation or GN-associated mortalities. In contrast, throughout the 10-mo course of identical, repeated R595 treatment, the C3H/HeJ mice had neither abnormal BUN nor histological evidence of renal disease. By immunofluorescence, the intensity of Ig deposits along the glomerular capillaries of the various groups corresponded in general to the extent of GN documented by light microscopy.

Effect of Heparinization in the R595-treated Mice. After injection of heparin, the whole blood clotting time of the R595 treated and nontreated mice was >12 h vs. a

TABLE III

Serum Levels of IgM and IgG Subclasses in R595-treated Mice and Unmanipulated Controls (5 Mo of Age)

	R595 Treatment	IgG3		IgG1		IgG2a		IgG2b		IgM	
						mg/ml					
MRL/n♀	+	9.6	(48)*	2.6	(1)	1.6	(1)	3.7	(5)	1.1	(2)
	−	0.2		1.9		1.1		0.7		0.5	
BXSB ♀	+	3.7	(19)	2.4	(1)	1.5	(1)	9.1	(10)	1.7	(9)
	−	0.2		1.8		1.3		0.9		0.2	
NZW♀	+	4.2	(21)	1.9	(1)	3.0	(2)	3.8	(8)	1.7	(5)
	−	0.2		1.7		1.3		0.5		0.3	
BALB/c♀	+	10.5	(21)	3.6	(2)	1.8	(1)	5.2	(8)	1.2	(6)
	−	0.5		1.7		1.4		0.7		0.2	
C57BL/6♀	+	0.5	(12)	1.7	(2)	1.4	(1)	11.0	(13)	1.25	(10)
	−	0.2		0.9		0.9		0.8		0.18	
C3H/HeJ♀	+	0.8	(1)	0.7	(1)	0.9	(1)	1.4	(1)	0.7	(1)
	−	0.7		0.6		0.8		1.2		0.6	

* Measured as stimulation index (treated/untreated control); ratio rounded to nearest whole number.

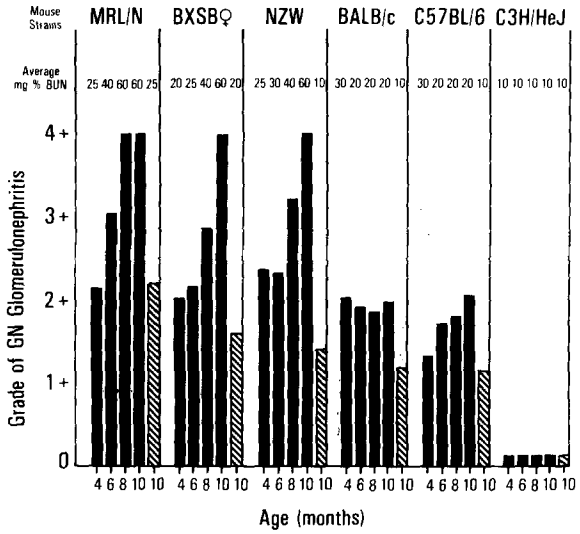

FIG. 1. Induction of GN in various strains of mice by prolonged stimulation with R595. Solid bars indicate the mean grade of GN present in the treated mice at each of the time intervals. Hatched bars represent the extent of GN seen in unmanipulated controls at 10 mo of age. Average BUN levels from these mice at the different time intervals are shown near the top of the figure. Each group includes 5–10 mice.

nonheparinized control time of 2–3 h. The anticoagulated R595 treated SLE-prone and normal mice developed serological and histological evidence of autoimmune disease at rates and severities similar to those of their nonanticoagulated counterparts (data not shown). Fibrinogen degradation products were not detected in the plasma of either the heparinized or nonheparinized R595-stimulated mice, when measured at 1, 2, and 24 h after injection, indicating absence of significant R595 endotoxin-

associated coagulopathy. By light microscopy and immunofluorescence, the amount of fibrinogen present in the diseased glomeruli of the R595-treated groups with and without heparin were similar, closely resembling those seen in the kidneys of moribund early SLE mice.

Induction of Other Histological Features of Autoimmunity. Table IV lists the incidences of other histological manifestations of autoimmune disease present in the R595 treated mice at autopsy. All mice except the C3H/HeJ had lymphoproliferation as indicated by the increased lymphoid mass in their spleens. In addition, the late-life SLE-prone mice (MRL/n and BXSB females) developed DVD and myocardial infarcts. Necrotizing arteritis occurred only in R595 treated MRL/n mice. The normal strains had no significant vascular or myocardial lesions.

Discussion

While several studies have shown that injections of LPS into normal mice can lead to production of autoantibodies and glomerular depositions of antigen-antibody complexes, including idiotype-antiidiotype forms (17), none of them have documented definitive histological or clinical evidence of immune complex disease, i.e., GN and vascular lesions. In a previous study, we have shown that a single injection of R595, the mitogenic lipid A portion of LPS, in normal mice (C57BL/6) induced a sustained polyclonal B cell activation with increased Ig and autoantibody production, both IgM and IgG classes, lasting at least 60 d. Our present study has extended these findings to demonstrate that prolonged in vivo polyclonal B cell activation with R595 will not only markedly accelerate the onset of autoimmunity and fatal immune complex disease in late-life SLE-prone murine strains, but will also lead to development of a limited form of autoimmune disease in normal mice. The forms, time of onset, and course of disease in the R595-treated late life SLE-prone mice resembled closely the spontaneous early SLE of their related MRL/l females, BXSB males, and NZB × NZW females.

Two recent studies of the effect of chronic LPS stimulation on SLE and normal mice carrying the *xid* gene defect have yielded differing results. Smathers et al. (18) found that chronic LPS administration (similar treatment to that used in this study)

TABLE IV

Incidence of Additional Parameters of Autoimmune Diseases in R595-treated Mice

	Lymphoproliferation*	DVD		Myocardial infarction	Vasculitis
		Incidence	Severity‡		
		%		%	%
MRL/n♀	2.3	88	3+	48	36
BXSB ♀	5.6	38	3+	31	0
NZW♀	3.0	80	1+	0	0
BALB/c♀	4.6	0	0	0	0
C57BL/6♀	3.3	0	0	0	0
C3H/HeJ♀	1.0	0	0	0	0

* Mean ratio increase of splenic white pulp, treated/untreated.

‡ Grading for DVD was done on a scale of 0 to 3+ based on examining four consecutive sections of individual bisected hearts. Grade 1 contained minimal PAS^+ deposits along and within one blood vessel wall; grade 2 showed PAS^+ deposits with lumenal narrowing in two or three coronary vessels; grade 3 contained four or more affected coronary blood vessels.

to NZB *xid*-bearing mice resulted in autoantibody production, whereas similarly treated DBA/2 *xid*-bearing mice did not make any autoantibodies. These findings were interpreted as evidence of the importance of genetic background (NZB vs. DBA/2) in the expression of autoimmunity, when exposed to certain environmental factors (chronic LPS stimulation). On the other hand, Steinberg et al. (19) reported that similarly treated (NZB × NZW)F_1 *xid*-bearing mice did not produce autoantibodies or autoimmune disease in spite of their obvious AID genetic background and suggested that the Ly-5^+ B lymphocytes (deleted in the *xid* gene effect) were essential in the early life SLE. In contrast, Ohsugi et al. (20) found that autoantibody production can occur in unmanipulated *xid*-bearing mice without obvious participation of the Ly-5^+ B lymphocyte subsets. These reports clearly indicate a lack of concensus about the roles of Ly-5^+ B lymphocytes (*xid* gene defect) and LPS in autoimmunity.

In the present study the distinctly different responses to R595 demonstrated by these three genetic/immunological backgrounds, i.e., late-life SLE-prone, normal, and nonresponder, emphasize the potential roles of polyclonal B cell activation and genetic predetermination in the genesis and expression of autoimmune SLE disease. R595, the lipid A-rich portion of LPS is in all likelihood a more effective chronic PBA than LPS since the latter is antigenic (21) and its efficiency as a chronic in vivo PBA is compromised by the generation of anti-LPS antibodies in the repeatedly injected mice, whereas no antibodies to R595 have been detected (22). Furthermore, the C3H/HeJ mice used in this study serve as more appropriate negative controls for LPS-R595 B cell activation than *xid*-bearing mice because they lack receptors for this activator but are not immunodeficient, and are fully capable of responding to other PBA (9). The absence of serological and histological features of AID in the chronically-treated C3H/HeJ mice seems to confirm that the R595 induced AID in the late-life SLE-prone and "normal" strains results specifically from activation of the immune system and not from other effects of endotoxin. In many respects, the R595 induced PBA acts similarly to the natural accelerating factors operating in mice with early SLE (23). The *lpr* gene of the MRL/l (24) or the Y chromosome-associated factor of the BXSB (25) are associated with early SLE not only in their usual hosts but also in any host with an AID genetic predisposition (23). The effect of R595 on the expression of AID is also almost identical to the effect of chronic lymphocytic choriomeningitis infection, which accelerates and makes more severe SLE in genetically predisposed mice and has relatively mild pathologic consequences in immunologically normal mice (26).

Whether T cell participation is required in this R595-induced autoimmunity remains speculative. While T cells are necessary in the adjuvant effect of LPS (27), R595 has been considered to be a T cell-independent PBA (28). In fact, in nude mice, R595 is postulated to replace and act as exogenous T cell help, leading to production of all IgG subclasses (29, 30). We also have preliminary results to indicate that in the absence of significant T cell populations (neonatal thymectomy), late-life SLE-prone and normal mice are capable of responding to R595 and developing autoimmune disease (data not shown).

Finally, several additional observations from this study of PBA induced autoimmunity are worthy of note:

(*a*) The accelerated SLE in the treated MRL/n mice is not accompanied by the

characteristic massive lymphadenopathy seen in their early SLE congenic MRL/l mice. This implies that the T lymphocyte proliferation and associated increased helper activity (31) in the latter can be bypassed or replaced by exogenous B cell activation leading to early and complete disease expression. In fact, neonatally thymectomized MRL/l mice treated with R595 developed accelerated autoimmunity and GN associated mortality comparable to the unmanipulated controls rather than enjoying the prolonged survival characteristic of neonatally thymectomized individuals (32).

(*b*) The selectivity of end-organ injury (other than GN) in these R595 treated late life SLE-prone mice very closely mimics their early SLE counterparts, again implying predeterminations by inherent genetic factors in the various manifestations of autoimmune disease. Arteritis, unique to the MRL/l mice (33), is found only in the treated MRL/n mice, whereas DVD and myocardial infarcts, occurring in ~20% of MRL/l and BXSB males (34), are detected in both the MRL/n and BXSB females treated with R595, though at a higher frequency. The treated normal mice, again, show a resistance to these autoimmune-mediated vascular and myocardial injuries, even though they manifest similar magnitudes of lymphoproliferation, Ig, and autoantibody production. These vascular and selective organ injuries could be related to subtle qualitative differences (i.e., affinity, subclass specificities, phlogogenicity) in the autoantibodies generated by the SLE-prone and normal mice. The qualitative differences of these autoantibodies are presently under investigation.

Summary

In vivo, prolonged polyclonal activation of B cells by the nonantigenic but potent mitogenic lipid A portion of lipopolysaccharide (LPS-R595) resulted in acceleration of the late life systemic lupus erythematosus disease of female MRL/n, BXSB, and NZW mice, mimicking the time, form, and histopathological features characteristic of their early life disease counterparts, i.e., MRL/l females, BXSB males, and (NZB × NZW)F_1 females. Similar polyclonal B cell activation of "immunologically normal" mice has less effect and led to a limited expression of autoimmune disease. This R595-induced autoimmunity and immune complex-mediated disease seemed to be the direct result of activation of the immune system and not from other effects of endotoxin since C3H/HeJ, a strain lacking lymphocyte receptors for LPS-R595, had neither serological nor histological evidence of autoimmune disease despite identical treatment.

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