against occurrence of autoimmune diseases by acting as a first-line defense molecule against infectious agents or by binding to apoptotic cells and cell debris, and thus contributing to clearance of potential autoantigens [18]. Further studies on the relationship between innate immunity functions and occurrence of type I diabetes may help understanding the pathogenesis of this condition.

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T cell epitopes of prothrombin in patients with antiphospholipid syndrome

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Antiphospholipid syndrome (APS) is characterised by arterial and/or venous thrombosis and recurrent fetal loss in association with the presence of antiphospholipid antibodies (aPL). In addition to β_2-glycoprotein I, prothrombin (PT) is an important autoantigen recognised by aPL. PT is a coagulation proenzyme abundantly present in blood (70–100 μg/ml), and binds to negatively charged phospholipids such as phosphatidylserine. PT comprises two major domains, fragment-1 (F-1) and prethrombin-1 (Pre-1). We previously reported that antiprothrombin antibody (aPT) is a mixture of antibodies against both F-1 and Pre-1, and that there are significant clinical differences between anti-F-1 and anti-Pre-1.[1 2]

aPL, including aPT, are not mere serological markers of the disease, but are important players in the pathogenesis of APS. Therefore, antigen-specific immunosuppressive treatments, if developed, will help to prevent thrombosis in APS. We aimed to locate the T cell epitopes of PT on either F-1 or Pre-1, as an initial approach to understanding the mechanism of aPT formation, and to search for possible PT-specific immunosuppressive treatments.

Samples from 15 patients with systemic lupus erythematosus (SLE) were positive for aPT. Fourteen patients were taking prednisolone at a mean of 10.8 mg/day (range 5.0–22.5). For T cell proliferation assays, samples from nine healthy volunteers served as controls.

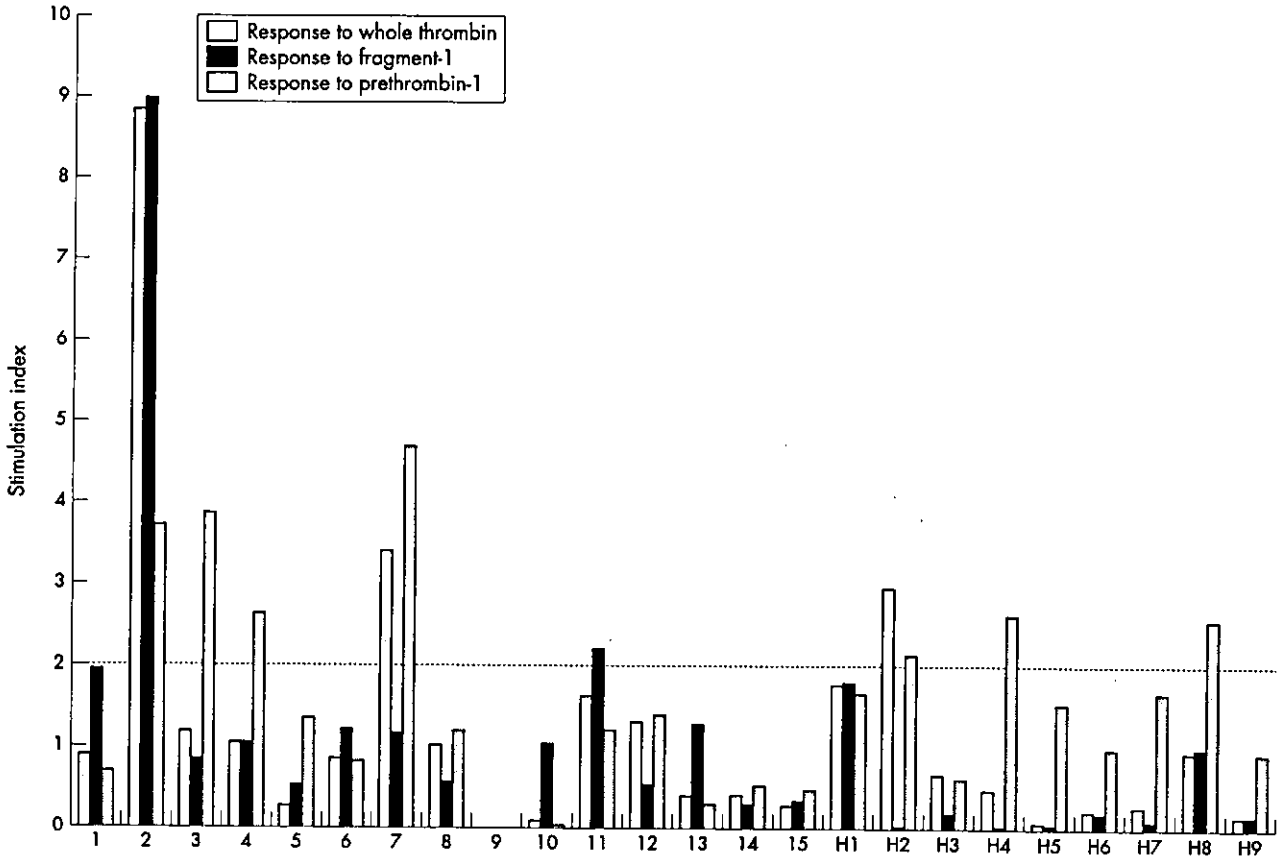

Figure 1 Proliferation of T cells stimulated by prothrombin. 1–15: subjects positive to antiprothrombin antibody; H1–H9: healthy controls. An SI≥2 was considered positive.

Table 1 Location of T and B cell epitopes on prothrombin, and HLA-DRB1 allotypes in patients positive for antiprothrombin antibodies

Patient	Age/sex	Diagnosis	Symptom	$a\beta_2GPI$	LAC	BFP	B cell epitope		T cell epitope		HLA-DRB1
							F-1	Pre-1	F-1	Pre-1	
1	53/F	APS/SLE	FL	+	–	+	+	–	+	–	*0101/*0901
2	60/F	SLE	Plt↓	+	–	+	+	–	+	+	*1502/*1101
3	29/F	SLE	–	–	+	–	+	–	–	+	*1502/*0803
4	39/F	SLE	–	–	–	+	+	–	–	+	*1101/*0901
5	40/F	SLE	FL	–	–	–	+	–	–	–	*1201/*0901
6	31/F	SLE	–	–	+	–	+	–	–	–	*0406/*1302
7	56/F	SLE	–	–	–	–	–	+	–	+	*1501/*0802
8	68/F	SLE	T	–	–	–	–	+	–	–	*1302/*1302
9	26/F	SLE	Plt↓	–	–	–	–	+	–	–	*1501/*0901
10	30/F	SLE	Plt↓	–	–	–	–	+	–	–	*1501/*1501
11	67/F	SLE	–	–	–	–	+	+	+	–	*0101/*0803
12	31/F	SLE	T, Plt↓	–	–	–	+	+	–	–	*1501/*1402
13	60/F	SLE	–	–	–	–	+	+	–	–	*14B/*1201
14	39/F	SLE	Plt↓	–	–	–	+	+	–	–	*0802/*0401
15	42/F	SLE	Plt↓	–	–	–	+	+	–	–	*1501/*1405

SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; FL, recurrent fetal loss; T, thrombosis; Plt↓, thrombocytopenia; $a\beta_2GPI$, anti-$\beta_2$glycoprotein I; LAC, lupus anticoagulant (determined by dilute Russell's viper venom time ratio); BFP, biological false positive for serological test for syphilis; F-1, fragment-1; Pre-1, prethrombin-1.

Prothrombin was cleaved as described[3] with some modifications. Briefly, 1 ml (1.46 mg/ml) of purified human PT (Haematologic Technologies, Essex Junction, VT) was incubated with 3 U bovine thrombin (Itoham Foods, Osaka, Japan) for three hours at 37°C. Cleaved products were run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, cut out, and purified using a model 422 Electro-Eluter (Bio-Rad, Hercules, CA).

Anti-PT, anti-Pre-1, and anti-F-1 enzyme linked immunosorbent assays (ELISAs) were performed as described previously.[3]

For T cell proliferation assays, peripheral blood mononuclear cells (2.0×10^5 cells/well) were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% L-glutamine-penicillin-streptomycin (Sigma), in the presence of PT, F-1 , Pre-1 (1 μmol/l) or controls, for three days under standard conditions. Antigen induced T cell proliferation was assayed by a cell proliferation ELISA, BrdU (Roche Diagnostics, Mannheim, Germany). The stimulation index (SI) was calculated as the mean optical density (OD) of wells containing the antigen/mean OD of the wells without antigen. An SI ≥ 2 was considered positive.

Samples from only two patients showed positive responses to PT. Because a large amount of PT is present in sera, T cells may be rendered tolerant in normal conditions, or some modifications may be necessary for PT to be incorporated by antigen presenting cells. On the other hand, three samples showed positive responses to F-1, and four to Pre-1, indicating the presence of at least one T cell epitope in those domains (fig 1). Among the six patients with positive T cell responses, the putative localisation of T and B cell epitopes was on the same domain in four patients, but was on different domains in two patients, suggesting that the location of T cell epitopes and the production of pathogenic aPT are not necessarily related (table 1).

HLA-DRB1*1501, which is suggested to be related to SLE,[4] was present in 5/15 (33%) patients. No significant skewing of the HLA-DRB1 alleles, or relationships with T cell epitopes were seen.

Samples from three controls showed positive responses to PT, F-1, or Pre-1. Autoantigen-specific T cells do exist in the sera of healthy subjects,[5] and the presence of autoantigen reactive T cells by itself is not sufficient to cause autoimmune diseases, other factors are necessary.

Our study shows that PT-specific T cells present in aPT positive patients do not recognise a single common T cell epitope, and that T cell epitopes are different among individual patients.

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Low Prevalence of Antibodies to Glucose-6-Phosphate Isomerase in Patients With Rheumatoid Arthritis and a Spectrum of Other Chronic Autoimmune Disorders

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Objective. **Arthritis in the K/BxN mouse model results from pathogenic immunoglobulins that recognize glucose-6-phosphate isomerase (GPI), a glycolytic enzyme residing in the cytoplasm of all cells. Antibodies directed against GPI can, alone, transfer arthritis to healthy recipients. Previous experiments have revealed significant titers of anti-GPI antibodies in the serum of many patients with rheumatoid arthritis (RA). We evaluated the generality of these observations in cohorts of patients with 12 different arthritic and chronic autoimmune diseases and in population-matched healthy control subjects.**

Methods. **Anti-GPI antibodies were assayed in 811 individual serum samples by enzyme-linked immunosorbent assay with 2 forms of GPI, recombinant and native. Results were confirmed by immunoblotting.**

Results. **Several patients had significantly elevated anti-GPI antibody titers, but without the prevalence or the specificity reported previously. Only 15% of RA patients had anti-GPI antibodies (range 12–29% in different cohorts), with a higher prevalence in patients with active disease. Psoriatic arthritis, undifferentiated arthritis, and spondylarthropathy patients also displayed anti-GPI antibodies at similar frequencies (12–25%). Similar titers were detected in a proportion (5–10%) of control subjects or patients with Crohn's disease or sarcoidosis. Very high titers were found in rare cases of RA and systemic lupus erythematosus.**

Conclusion. **No disease-specific pattern of antibody positivity to GPI was apparent. While the antibody-mediated mechanism at play in the mouse model may exemplify a generic mechanism for some forms of arthritis in humans, GPI itself does not appear to be a target common to the majority of RA patients.**

The pathogenesis of arthritis, in particular rheumatoid arthritis (RA), is, for the most part, poorly understood. In particular, the role of joint-specific autoimmunity remains a subject of controversy. It is not known, for example, whether T cells are dominant players in the local inflammation or whether they act

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upstream in the process by helping B cells produce pathogenic antibodies (1–3). Studies of the K/BxN mouse, a recently described model of arthritis, have shown that the full scope of arthritic manifestations can be elicited by antibodies.

Similar to RA in humans, the spontaneous disease in this model requires a particular class II allele of the major histocompatibility complex, is chronic, progressive, and symmetric, and results in severe destruction of cartilage and bone. A classic histologic complex of synovitis, leukocyte invasion of the articular cavity, and pannus formation leads to cartilage and bone destruction and is dependent on inflammatory cytokines such as interleukin-1 and tumor necrosis factor α (4).

The immunologic phase of the disease is characterized by the generation of arthritogenic autoantibodies, which is provoked by the autoreactivity—and only partial tolerization—of T lymphocytes expressing the transgenic T cell receptor (5). However, once the pathogenic antibodies have been elicited by the autoimmune reaction, there is no further requirement for lymphoid cells, and the antibodies alone can transfer full-blown arthritis to naive lymphocyte-deficient hosts (5,6). Studies of the K/BxN model also indicated that ubiquitous antigens can be the target of arthritogenic antibodies: those in K/BxN mice recognize the widespread glycolytic enzyme glucose-6-phosphate isomerase (GPI) (7–9).

Does GPI also serve as a target in human diseases? Our initial analyses did not show a high level of anti-GPI antibodies in the sera of RA patients (Korganow AS, Benoist C, Mathis D: unpublished observations). Yet, very strikingly, Schaller et al (10) reported a high frequency of such antibodies in RA patient sera, but not in sera from patients with Sjögren's syndrome (10), although those results have been the subject of debate (11–13). In addition, GPI deposits can be detected on sections of inflamed synovium from RA patients, much as they can in tissues from the joints of K/BxN mice (9,10). Thus, it seemed important to further evaluate the prevalence of antibodies to GPI in a broad range of arthritic conditions compared with other chronic autoimmune/inflammatory diseases.

PATIENTS AND METHODS

Patient cohorts. Patients of the Boston cohort (n = 82) were outpatients under the care of a qualified rheumatologist at the Robert B. Brigham Arthritis Center, Brigham and Women's Hospital (Institutional Review Board protocol no. 2001-P-001620). Medical records were reviewed for signs and symptoms that met the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for the classification of rheumatic diseases, including RA (14), psoriatic arthritis (PsA) (15), and juvenile rheumatoid arthritis (JRA) (16), and the European Spondylarthropathy Study Group (ESSG) criteria for spondylarthropathy (SpA) (17). The date of disease onset and other laboratory, radiographic, therapeutic, and demographic data were also identified. Laboratory values included the erythrocyte sedimentation rate (ESR; highest value) and rheumatoid factor (RF; titer or unit value). Available radiographs were predominantly of the hands and feet, and had been interpreted by a certified radiologist. All control subjects were free of rheumatic diseases.

The Winnipeg cohort (n = 105) was recruited from patients of community physicians in the Winnipeg area. All were evaluated clinically and followed up at the University of Manitoba Arthritis Center (University of Manitoba Institutional Review Board protocol no. B2001-070). Patients met the ACR criteria for RA (14) or the ESSG criteria for SpA (17); patients with viral arthritis had clinical syndromes typical of these diagnoses. The duration of symptoms in all but 4 patients was between 3 and 12 months. Except for the 4 patients with established RA, none of the patients had taken any disease-modifying antirheumatic drugs (DMARDs). All control subjects were free of rheumatic disease symptoms, and were derived from the same geographic area as the patients.

For the Tsukuba cohort (n = 156), patients with autoimmune disease were randomly recruited from patients attending the autoimmune disease clinic at Tsukuba University Hospital (approved protocol entitled "Research for Detecting Susceptible Genes for Autoimmune Diseases"). The number of tender joints was determined in 49 peripheral joints, and the number of swollen joints was determined according to the presence of effusion and/or synovial thickening in 46 joints (excluding the neck and hips). Erosions identified on radiographs of affected joints at the time of sampling were recorded. The ACR criteria for RA (14) and systemic lupus erythematosus (SLE) (18), and the criteria of the Sjögren's Disease Research Committee of the Ministry of Health and Welfare in Japan (19) were applied. All control subjects were free of rheumatic diseases.

For the National Institutes of Health (NIH) cohort (n = 225), patients with inflammatory arthritis of <1 year's duration and involving ≥ 1 swollen joint were enrolled into a study of early synovitis at the National Institute of Arthritis and Musculoskeletal and Skin Diseases/NIH (protocol no. 94-AR-194). The number of swollen joints was determined by evaluating patients for the presence of effusion and/or synovial thickening in 66 peripheral joints (hips were excluded). Anteroposterior and lateral radiographs of the hands and feet were obtained at the initial visit and at the 1-year followup visit and were evaluated for the presence of erosions. The ACR criteria for RA (14) and the ESSG criteria for SpA (17) were applied. Patients who did not fulfill either set of criteria were classified as having undifferentiated arthritis. Sera obtained from the initial visit were assessed for a panel of arthritis-associated autoantibodies including anti-Sa, antifilaggrin, anticitrulline, and antikeratin as described (20).

Sera from a cohort of patients with nonarticular chronic autoimmune/inflammatory diseases who were receiving care at the Hôpitaux Universitaires de Strasbourg in Strasbourg, France (n = 60), were also studied. These patients

had a histologically confirmed diagnosis of either sarcoidosis (some with systemic extrapulmonary manifestations) or Crohn's disease.

Enzyme-linked immunosorbent assay (ELISA). All assays were performed in Boston on samples shipped frozen from the participating centers. Recombinant human GPI (rHuGPI) was prepared using the plasmid pGEX-4T3 (Pharmacia, Piscataway, NJ) in which the 16–1692 fragment of human GPI complementary DNA had been inserted (positions from the K03515 sequence). The resulting clone encodes amino acids 1–559 of GPI (the complete coding sequence) linked to glutathione S-transferase (GST) via a thrombin-cleavable linker. Recombinant protein was prepared by growing *Escherichia coli* harboring the pGEX-hGPI plasmid in L-broth (containing 100 μg/ml of ampicillin) at 37°C to an optical density (OD) at 550 nm of 0.6. After growth, 0.1 m*M* IPTG was added to the medium, and bacterial culture was continued overnight at 20°C.

Cells were centrifuged, the cell pellet was suspended in Buffer A (50 m*M* Tris HCl, pH 8.0, 0.5 m*M* EDTA, 0.4*M* NaCl, 5 m*M* $MgCl_2$, 5% glycerol, 10 μg/ml of aprotinin, and 0.1 m*M* dithiothreitol) containing 1 mg/ml of lysozyme. Samples were kept on ice for 1 hour, then frozen and thawed twice. After the addition of EDTA (1 m*M* final concentration) and Nonidet P40 (0.5% volume/volume), the cell suspension was sonicated 5 times for 1 minute on ice, and cleared by centrifugation. The resulting solution was loaded onto a glutathione–Sepharose column (Pharmacia) equilibrated in phosphate buffered saline (PBS). The column was washed with 10 bed volumes of PBS. The GST-hGPI fusion protein was then eluted with 10 bed volumes of elution buffer (5 m*M* glutathione, 50 m*M* Tris HCl, pH 8.0). The amounts and purity of the protein were estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

This preparation or rabbit muscle GPI (rabGPI; ~50% full-length as estimated by SDS-PAGE) (Sigma, St. Louis, MO) was used at 5 μg/ml (diluted in PBS) to coat microtiter plates (Costar 9017) for 12 hours at 4°C. After washing twice with washing buffer (0.05% Tween 20 in PBS), 2% skim milk (Stop & Shop, Brookline, MA) in PBS was used for blocking of nonspecific binding (30 minutes at 37°C). After 2 washes, sera (diluted 1:100 in essentially all experiments) were added, and the plates were incubated for 12 hours at 4°C or for 2 hours at 37°C. After washing, alkaline phosphatase (AP)–conjugated anti-human IgG (Fc fragment specific; Jackson ImmunoResearch, West Grove, PA) was added to the plates (1:1,000 dilution) for 1 hour at room temperature. After 3 washes, color was developed with AP reaction solution (containing 9.6% diethanolamine, 0.25 m*M* $MgCl_2$, pH 9.8, with AP substrate tablets [1 AP tablet per 5 ml of AP reaction solution]; Sigma). Plates were incubated for 1 hour at room temperature, and the $OD_{405\ nm}$ was measured by plate spectrophotometry. Determinations were performed in triplicate and standardized between experiments by reference to a highly positive human anti-GPI serum.

Anti-GPI reactivity to each antigen was corrected by subtracting the OD readings for anti-GPI reactivity in parallel wells coated with matched molar amounts of GST (for rHuGPI) or bovine serum albumin (BSA; for rabGPI). OD values for the controls were usually in the range of 0.26–0.62 and 0.13–0.21, respectively. Approximately 5% of sera exhibited high reactivity (>1.0) to GST alone. These OD values were tabulated and then converted to 2 anti-GPI indices. Index 1 combined the reactivity against either antigen, and was calculated as follows:

$$\text{Index } 1(i) = \sqrt{(x_i^2 + y_i^2)}$$

where x_i and y_i are the OD readings for rabGPI and rHuGPI of serum i, respectively. Index 2 considered only the shared reactivity against both antigens and was calculated as follows:

$$\text{Index } 2(i) = \min(x_i, y_i)$$

where min represents minimum.

For the competitive ELISA, patient sera were diluted to yield similar anti-GPI OD readings on rHuGPI-coated plates. Several wells were set up in parallel, with no or with graded amounts of soluble rHuGPI (5–100 μg/ml) added to rHuGPI-coated plates, followed immediately by the addition of patient sera. Plates were then incubated for 2 hours at 20°C. The next steps of the ELISA were performed as described above.

Immunoblot analysis. To prepare human GPI that was free of the GST moiety, rHuGPI-GST was digested by thrombin according to the manufacturer's protocol (Pharmacia). Preliminary gel electrophoresis showed these preparations to be >90% digested. The digestion mixture (still including the 26-kd GST fragment) was separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Jupiter, CA). After blocking with 5% weight/volume skim milk in PBS (12 hours at 4°C), the membrane was cut into thin strips. Each strip was incubated with 1 ml of patient serum diluted 1:100 in PBS supplemented with 5% w/v skim milk powder (2 hours at 20°C). Bound antibody was visualized with AP-conjugated anti-human IgG (Jackson ImmunoResearch), and visualized with AP reaction solution containing 0.4 m*M* nitroblue tetrazolium (Bio-Rad) and 0.4 m*M* 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt (Bio-Rad) for 3 minutes at room temperature.

RESULTS

Findings in the patient cohorts. In order to investigate the broadest possible group of patients, we analyzed several independent cohorts. The characteristics of each of the cohorts are as follows.

The Boston cohort (n = 119) provides a cross-disease representation of patients receiving followup care at the Brigham and Women's Hospital Arthritis Center (Table 1). Disease duration at the time of serum collection varied, ranging from 0.2 years to 32 years. These patients were categorized by clinician's assessment as having "inflammatory" (presenting with acute or ongoing inflammatory manifestations at the time of serum collection) or "stable" (controlled disease; not very active at the time of sampling) disease. A variety of treatments were being given at the time of sampling; 79% were being treated with at least 1 DMARD. The

Table 1. Demographics of the Boston cohort*

	Healthy controls (n = 37)	RA		PsA (n = 17)	JRA (n = 4)	SpA (n = 6)
		RF+ (n = 41)	RF− (n = 14)			
Age, mean ± SD (range) years	44 ± 15 (23–84)	55 ± 17 (17–81)	57 ± 17 (28–73)	43 ± 12 (24–50)	29 ± 6.5 (23–38)	35 ± 10 (24–50)
% female	63	84	93	59	50	0
Symptom duration, mean years	–	9.0	7.0	6.8	17.1	8.23
ESR, mean ± SD mm/hour	–	60 ± 25	57 ± 37	46 ± 31	76 ± 42	48 ± 27
% with RF >15 units	–	100	0	0	100	0
% with erosions	–	74	50	25	67	40
% taking steroids	–	45	57	21	75	17
% taking DMARDs	–	88	79	86	75	67

* Radiographs were available for determination of erosions in only 61 of the 82 patients. RA = rheumatoid arthritis; RF = rheumatoid factor; PsA = psoriatic arthritis; JRA = juvenile rheumatoid arthritis; SpA = spondylarthropathy; ESR = erythrocyte sedimentation rate; DMARDs = disease-modifying antirheumatic drugs.

control group (n = 37) was collected at the Joslin Diabetes Center, largely from the same New England population, and consisted primarily of healthy partners of diabetic patients as well as a subset of laboratory workers. These subjects were free of autoimmune disease.

The Winnipeg cohort (55 affected and 50 controls) was derived primarily from an early synovitis clinic at the University of Manitoba Arthritis Centre in Winnipeg (Table 2). Patients varied with regard to disease duration at the time of serum collection.

The Tsukuba cohort (n = 205) provides a cross-disease presentation of Japanese patients randomly chosen from patients receiving followup care at the Autoimmune Disease Clinic of Tsukuba University Hospital (Table 3). The diagnoses included RF-positive RA (n = 70), seronegative RA (n = 28), SLE (n = 38), and Sjögren's syndrome (n = 20). Patients varied with regard to disease duration at the time of serum collection, and they were receiving a diverse range of treatments. This cohort also included both active and stable forms of disease at the time of sampling. The healthy donor samples (n = 49) were collected from hospital and laboratory volunteers at Tsukuba University.

The NIH cohort (n = 225) represents a set of patients whose serum was sampled close to the onset of their arthritis, with synovitis of <1 year's duration (Table 4). The diagnoses included seropositive RA (n = 71), seronegative RA (n = 34), SpA (n = 40), and undifferentiated arthritis (n = 80). While a healthy control group was not included, this cohort is interesting because it has already been thoroughly characterized for the association of synovitis with a panel of autoantibodies (20), permitting a test of the correlation of anti-GPI antibodies with other RA-related antibody specificities.

Nonarticular chronic autoimmune/inflammatory diseases were represented by a set of sera, all sampled at the time of initial presentation, from the Hôpitaux

Table 2. Demographics of the Winnipeg cohort, all of whom had disease of recent onset*

	RA		PsA (n = 3)	Arthralgias (n = 16)	Viral (n = 3)
	RF+ (n = 31)	RF− (n = 2)			
Age, mean (range) years	45 (16–76)	59 (46–73)	28 (20–37)	42 (20–63)	30 (17–39)
% female	60.7	100	66.7	73.3	66.7
% Caucasian	66.7	100	66.7	81.3	100
Tender joint count, mean ± SD	12.6 ± 10	23	10 ± 4.4	9.8 ± 14.2	0
Swollen joint count, mean ± SD	10 ± 7.5	24	3.3 ± 2.1	3 ± 4	8
ESR, mean ± SD mm/hour	24.4 ± 26	26.5 ± 20	38.3 ± 47	7.5 ± 6	11 ± 9
CRP, mean ± SD mg/dl	17.7 ± 25	8 ± 5.7	39.6 ± 61	4.3 ± 0.9	4
% taking prednisone	6	50	0	0	0
% taking DMARDs	9.6	50	0	6.3	0

* This cohort includes 50 population-matched controls. RA = rheumatoid arthritis; RF = rheumatoid factor; PsA = psoriatic arthritis; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs.

Table 3. Demographics of the Tsukuba cohort*

	Healthy controls (n = 49)	RA RF+ (n = 70)	RA RF− (n = 28)	SLE (n = 38)	SS (n = 20)
Age, mean ± SD years	29 ± 8	58 ± 13	43 ± 17	50 ± 13	54 ± 11
% female	66	89	65	90	90
Symptom duration, mean ± SD months	–	129 ± 144	128 ± 119	171 ± 105	84 ± 102
Tender joint count, mean ± SD	–	5 ± 3	4 ± 3	0.1 ± 0.3	0
Swollen joint count, mean ± SD	–	4 ± 3	3 ± 3	0.1 ± 0.3	0
ESR, mean ± SD mm/hour	–	53 ± 32	30 ± 17	14 ± 4	47 ± 18
CRP, mean ± SD mg/dl	–	1.8 ± 2.2	1 ± 1.2	0.3 ± 0.5	0.4 ± 0.9
% with RF >20 units	–	100	0	20	90
% with erosions	–	64	62	0	0
% taking prednisone	–	54	62	90	0
% taking DMARDs	–	82	75	0	0

* The number of tender joints was determined in 49 peripheral joints; the number of swollen joints was determined according to the presence of effusion and/or synovial thickening in 46 joints (excluding the neck and hips). Erosions were identified on radiographs of affected joints taken at the time of sampling. RA = rheumatoid arthritis; RF = rheumatoid factor; SLE = systemic lupus erythematosus; SS = Sjögren's syndrome; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs.

Universitaires de Strasbourg (Strasbourg, France). Twenty patients presented with a histologically confirmed diagnosis of sarcoidosis (mean age 40 years, range 27–59 years), of which 11 had systemic extrapulmonary manifestations. Forty patients had a diagnosis of Crohn's disease that had been confirmed histologically (mean age 33, range 19–44 years). Sera from these patients were also sampled at the time of diagnosis (32 patients) or at the time of an acute disease flare (8 patients).

For all cohorts, the sera were aliquoted shortly after collection (within 8 hours in almost all instances), and were frozen at −80°C; aliquots were thawed and refrozen, at most, only once after this. Significant problems with the reproducibility of the anti-GPI assay results occurred with sera that had been frozen and thawed multiple times or sera that had been kept for long periods of time at 4°C (data not shown). It is possible that the GPI present in the serum samples, albeit at a low level, may influence the stability of the anti-GPI reactivity under such conditions.

Validation of technical choices for the ELISA. The initial report of anti-GPI reactivity in RA sera elicited objections to the validity of the antigen used in the ELISA or the specificity of the assay conditions (11–13). We tested a number of experimental variables,

Table 4. Demographics of the National Institutes of Health cohort*

	RA RF+ (n = 71)	RA RF− (n = 34)	SpA (n = 40)	UA (n = 80)
Age, mean ± SD years	47 ± 12	44 ± 14	37 ± 11	41 ± 14
% female	60	74	63	7
% Caucasian	77	92	100	80
Symptom duration, mean ± SD weeks	33 ± 17	30 ± 24	31 ± 36	35 ± 37
Tender joint count, mean ± SD	19 ± 12	22 ± 12	5 ± 10	5 ± 5
Swollen joint count, mean ± SD	13 ± 9	16 ± 11	2 ± 2	3 ± 3
ESR, mean ± SD mm/hour	45 ± 28	41 ± 31	40 ± 30	33 ± 30
CRP, mean ± SD mg/dl	1.8 ± 1.8	1.8 ± 2.0	1.9 ± 3.2	1.5 ± 1.9
% with RF >20 units	100	0	5	12 (15)
% with erosions	45	36	12	16
% taking prednisone	34	44	25	12
% taking DMARDs	38	35	18	13

* The number of swollen joints was determined by evaluating patients for the presence of effusion and/or synovial thickening in 66 peripheral joints (hips were excluded). Radiographs were available for determination of erosions in only 183 of the 225 patients. RA = rheumatoid arthritis; RF = rheumatoid factor; SpA = spondylarthropathy; UA = undifferentiated arthritis; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs.

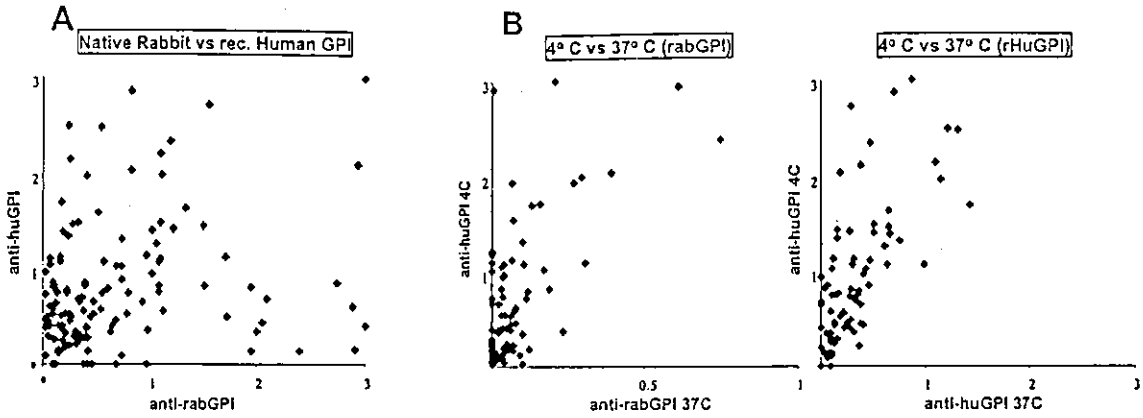


Figure 1. Enzyme-linked immunosorbent assay (ELISA) conditions for anti-glucose-6-phosphate isomerase (anti-GPI) determination. A, Reactivity of human sera (Boston cohort; different diagnoses pooled) against both recombinant (rec.) human GPI (anti-huGPI; y-axis) and purified rabbit muscle GPI (anti-rabGPI; x-axis). ELISAs were performed against each antigen as described in Patients and Methods, with sera diluted 1:100. Each data point represents the optical density (OD) value for a single serum sample (corrected by subtracting background reactivity against bovine serum albumin or glutathione S-transferase, respectively). B, Comparison between reactivities at 4°C (4C) and 37°C (37C). ELISAs were performed with either rabbit GPI (left) or recombinant human GPI (right), and the data were processed as for A. Each data point represents a single serum sample.

which, given the divergent views, are worth detailing here. We utilized 2 forms of antigen, thus guarding against problems with spurious cross-reactivity or contaminants in one or another preparation.

First, we adapted our previous ELISA (7) with full-length rHuGPI, expressed in *E coli* as a GST fusion protein, and purified to homogeneity by affinity chromatography on glutathione–Sepharose (>90% purity on SDS-PAGE, devoid of any major contaminant). Free GST purified in parallel was used as a control. Second, because this recombinant form of GPI may not have the same conformation or posttranslational modifications as the natural enzyme, we also utilized native GPI purified from rabbit liver. This is also the antigen used by Schaller et al (10), thus allowing a direct comparison with their results. While not strictly identical in amino acid sequence to the human protein (93% identity), this form of GPI provides a native conformation of the enzyme with normal posttranslational modifications. SDS-PAGE showed this preparation to contain ~50% GPI, as previously reported (12). BSA was used as the negative control in this instance.

As shown in Figure 1A, reactivity to both forms of GPI was not always correlated. Some sera did show reactivity to both, but a significant proportion reacted very preferentially to one or the other of the antigens (in contrast, serum from arthritic K/BxN mice reacted vigorously to both forms, even at high dilutions). Thus, we systematically tested both forms of antigen. On the other hand, the overall conclusions concerning association with disease proved similar with either antigen source (see below), and for tabulations of reactivity frequencies, we calculated combined indices, integrating reactivity to either or both antigens (see Patients and Methods).

Assay conditions were also tested systematically. The optimal concentration of coating antigen was tested with K/BxN serum as a guide, and a number of blocking agents were tested, lest one lead to a higher background value through adventitious reactivity. In several assays, we found no significant difference in background reactivity levels when using BSA (1–4%), commercial skim milk (2–5%), or horse serum (5%) as blocking agents (data not shown). Because Schaller et al (10) had used incubations with test sera at 37°C, rather than our incubation temperature of 4°C, we also tested the 2 temperatures in parallel. As shown in Figure 1B, there was no significant difference between the 2 conditions, and the results largely correlated (with a higher overall binding at 4°C, as might be expected). In the full analysis, binding was tested at both temperatures for almost all sera, with very similar conclusions (but, for simplicity, only the results obtained at 4°C are shown below).

All sera were tested in parallel at a dilution of 1:100, using for standardization a serum pool from arthritic K/BxN mice, which allowed comparisons between experiments. The primary readings were pro-

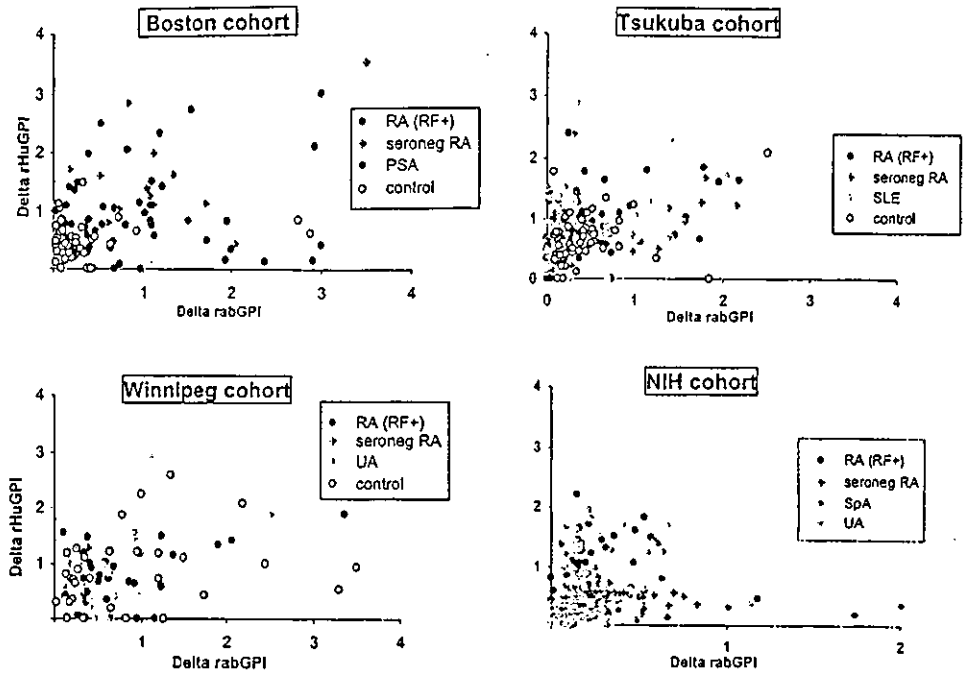


Figure 2. Enzyme-linked immunosorbent assays were performed with both rabbit glucose-6-phosphate isomerase (rabGPI) and recombinant human GPI (rHuGPI) as targets, and the data were corrected by subtraction of background values, as described in Figure 1. The assay conditions were maintained constant between experiments, using for a constant reference a pool of K/BxN mouse sera. Each data point represents an individual patient; the diagnoses are color-coded as indicated. RA = rheumatoid arthritis; RF+ = rheumatoid factor positive; seroneg = seronegative; PsA = psoriatic arthritis; SLE = systemic lupus erythematosus; SpA = spondylarthropathy; UA = undifferentiated arthritis.

cessed by subtracting OD readings of control wells (coated with GST and BSA for rHuGPI-GST and rabGPI, respectively). The values were not processed further, even though linearity did drop off at high OD values, leading to log-like compression of the readings. As will be seen below, further processing would not have substantially altered the basic conclusions on the relative distribution of values in the patient and control groups.

ELISA detection of anti-GPI reactivity. Combined ELISA results for the 811 sera are displayed in Figure 2, where reactivities against rabGPI and rHuGPI are shown separately for each cohort and are color-coded for disease status. In the different cohorts, there was no strikingly different distribution of anti-GPI reactivities in patients with RA, patients with other arthritic diseases, and controls. A continuous spectrum of values was observed, with no obvious cutoff between positive and negative sera; the large majority of samples yielded OD readings of ≤1.0 with either antigen. A few samples showed greater reactivity (>1.0) with either or both antigens. In the Boston cohort, and to some extent in the Tsukuba cohort as well, these included a greater proportion of patients with arthritis, but this was not necessarily RA. This was not confirmed in the Winnipeg cohort, where more than half of the sera with higher anti-GPI titers were from healthy control subjects.

Two "anti-GPI indices" were calculated for the entire data set, based on the reactivity to both rabGPI and rHuGPI antigens. The first index combines the reactivity against both antigens, since it is possible that unique epitopes are present on only one antigen form. On the other hand, since either antigen preparation might contain contaminating proteins that would confound the analysis (trace bacterial contaminants for the recombinant protein or other muscle proteins for the rabbit muscle GPI), the second index scores more favorably the shared reactivity against both antigens. For both of these indices, cutoff points for positivity were calculated as the 95% percentile of the values in control subjects.

The proportion of positive sera was calculated for the various diagnoses across all cohorts (Table 5). While there was a trend toward a higher frequency of reactivity in sera from arthritis patients, no measure clearly distin-

Table 5. Frequency of anti–GPI-positive sera in 811 patients with different pathologic conditions*

Diagnosis	% anti-GPI positive		Anti-GPI index		No. of sera
	Rabbit GPI	rHuGPI	Index 1†	Index 2‡	
Nonarthritic controls	5.3	5.3	7.0	6.1	114
Seropositive rheumatoid arthritis	5.4	13.0	14.6	7.5	239
Seronegative rheumatoid arthritis	9.3	9.3	15.5	11.3	97
Juvenile rheumatoid arthritis	10.5	5.3	21.1	25.0	19
Psoriatic arthritis	5.0	15.0	15.0	5.0	20
Spondylarthropathy	5.7	17.0	24.5	0.0	53
Systemic lupus erythematosus	2.6	7.7	7.7	5.0	39
Sjögren's syndrome	5.0	0.0	5.0	5.0	20
Viral arthritis	33.3	33.3	33.3	10.5	3
Undifferentiated arthritis	2.2	14.4	12.9	10.3	139
Undifferentiated arthritis (RF+)	12.5	0.0	12.5	15.0	8
Crohn's disease	5.0	5.0	7.5	33.3	40
Sarcoidosis	5.0	10.0	10.0	7.6	20

* The proportion of positive sera (those above a cutoff defined as the 95% percentile of control sera) was calculated independently for optical density readings corrected against rabbit glucose-6-phosphate isomerase (GPI) and recombinant human GPI (rHuGPI) antigens. RF = rheumatoid factor.
† Anti-GPI index 1 combines the reactivity against both the rabbit and the recombinant human GPI antigens (as the square root of [(rabbit GPI)^2 + (rHuGPI)^2]), and positivity was determined with the same cutoff.
‡ Anti-GPI index 2 tracks the shared reactivity against both antigens (the minimum reading).

guished reactivities in RA patients. Neither the combined anti-rabGPI and anti-rHuGPI reactivities of index 1 nor the dual reactivities of index 2 highlighted any disease group. The slightly increased frequencies in RA patients were not specific to the disease, since sera from patients with PsA and with SpA exhibited similar frequencies. Patients with Crohn's disease showed the highest frequency of dual reactivity.

A very small subset of patients had considerably higher titers against both forms of GPI (detectable at dilutions of ≥1:5,000). Again, there was no disease specificity in this group, since these very high titers were found in 1 SLE patient, 1 seronegative RA patient, and 1 RA patient with severe extraarticular manifestations.

We attempted to correlate anti-GPI titers with disease parameters within the RA group. No association was found between anti-GPI reactivities and either the patient's sex, age at disease onset, or disease duration, the presence of RF (seronegative RA patients had a slightly higher prevalence of reactivity, particularly those in the Boston cohort) or radiographic erosions (the 3 RA sera with the highest anti-GPI titers were from patients with limited radiographic erosions), or the type of treatment. On the other hand, active disease correlated with somewhat higher anti-GPI antibody levels. As shown in Figure 3, patients with the more active forms of RA showed a higher prevalence of serum anti-GPI positivity (27.3% positive for anti-rabGPI, 18.2% positive for anti-rHuGPI, 36.4% for index 1, and 22.7% for index 2 in the "inflammatory" RA subgroup versus 6.1%, 15.2%, 24.2%, and 9.1%, respectively, in the "stable" RA subgroup; $P = 0.008$, $P = 0.19$, $P = 0.04$, and $P = 0.03$, respectively, by t-test).

Arthritogenic antibodies in K/BxN mice have a very high affinity for GPI, and it was conceivable that sera from patients with arthritis had anti-GPI antibodies of higher affinity than those from controls (21). In an attempt to address this question, we performed competitive ELISA experiments (higher affinity antibodies being more easily displaced from the target by low concentrations of soluble competitor). No significant

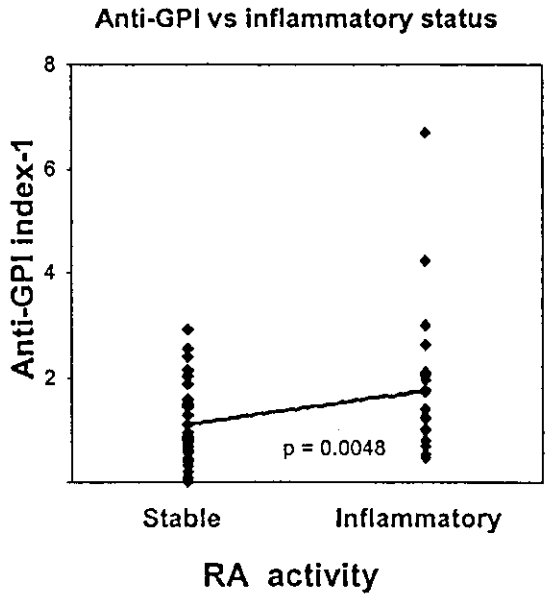
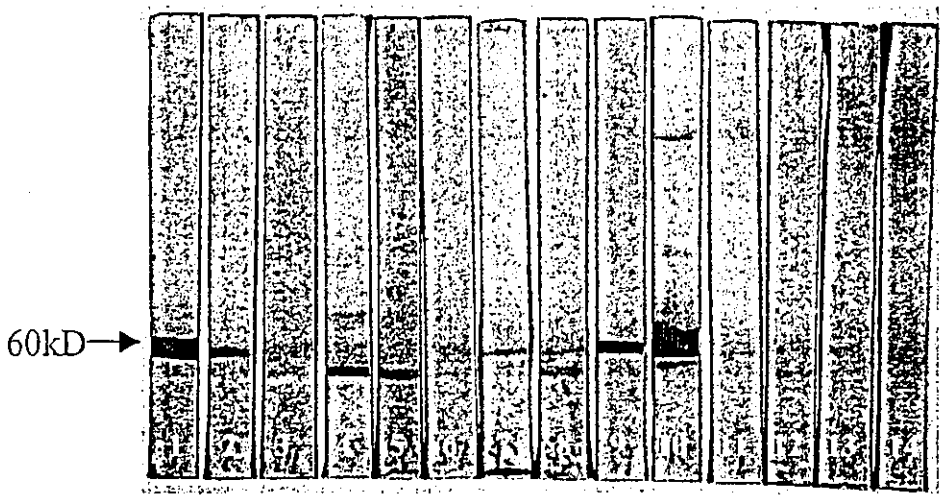


Figure 3. Correlation of higher anti–glucose-6-phosphate isomerase (anti-GPI) reactivities with inflammatory status. Rheumatoid arthritis (RA) patients (Boston cohort only; both seropositive and seronegative patients) were categorized by their disease status (determined by a rheumatologist prior to determining anti-GPI antibody values) at the time the serum sample was collected. Those with stable RA had long-term disease that was relatively controlled, with mainly dormant sequellar lesions; those with inflammatory RA had active inflammatory lesions at the time of sampling. Values are the anti-GPI index 1 data (see Table 5); each data point represents an individual patient.



rHuGPI O.D.	3.5	2.044	1.646	1.973	2.347	2.481	2.717	2.863	3		0.294	0.472	0.51	1.49
rabGPI O.D.	3.5	0.825	1.326	0.4	1.188	0.53	1.552	0.825	3		0.046	0.023	0.04	0.33
Diagnosis	SnRA	PSA	SnRA	RA	RA	RA	PSA	SnRA	RA	K/BxN	C	C	C	C

Figure 4. Correlation of anti–glucose-6-phosphate isomerase (anti-GPI) reactivities on enzyme-linked immunosorbent assay with reactivities on Western blots. Individual sera from patients with arthritis (lanes 1–9) were used to probe electroblot strips on which recombinant human GPI (rHuGPI; a whole gel was blotted, and strips were cut longitudinally) had been blotted. Lane 10 was probed with control serum from an arthritic K/BxN mouse; lanes 11–14 were probed with sera from healthy subjects. The position of GPI (60 kd) is indicated at the left. O.D. = optical density; rabGPI = rabbit GPI; SnRA = seronegative rheumatoid arthritis; PSA = psoriatic arthritis; RA = rheumatoid arthritis; C = control.

differences were observed between RA and control sera in these assays, but the results indicated that the anti-GPI antibodies present in human sera were of lower affinity than those present in sera from the K/BxN mouse model (data not shown).

The NIH cohort (Table 4) (20) is composed of sera from a clinical spectrum of patients with arthritis of recent onset. Sera from this cohort have been well characterized for antibodies that have previously been associated with RA: anticitrulline, antikeratin, antifilaggrin, and anti-Sa (22–25). As previously described, these antibodies were significantly associated with the diagnosis of RF-positive RA in this patient cohort ($0.00001 < P < 0.0005$) (20). However, antibodies to GPI showed no correlation with these RA-associated antibodies ($0.10 < P < 0.90$, by 2-tailed t-test) (data not shown). There was only a slight correlation between anti-rabGPI titers (but not anti-rHuGPI) and positivity for antinuclear antibodies ($P < 0.02$, corrected for multiple sampling).

Confirmation of anti-GPI reactivity by Western blotting. Questions have been raised about the purity of the commercial rabGPI preparation, with the suggestion by Schubert et al (12) that a proportion of the serum antibodies from their RA patients were reactive against contaminating creatine kinase. Although our study attempted to avoid such problems by combining measurements with 2 different antigens, it was nevertheless important to verify that the anti-GPI antibodies we detected were indeed reactive to GPI. This was verified by Western blotting, with strips of electroblot membrane onto which purified rHuGPI and rabGPI had been transferred after SDS-PAGE separation. Electroblotting affords the spatial resolution to distinguish true anti-GPI signals from reaction against contaminants, but would have been impractical to apply to the whole set of 811 samples. Analyses were therefore performed on subsets of patient and control sera that yielded OD readings >1.0 in the ELISA assays.

The example shown in Figure 4 confirms that the strong positive ELISA readings indeed corresponded to anti-GPI antibodies in the majority of cases (lanes 1, 2, 3, 6, 7, 8, 9, and 14). Some sera detected other bands (lanes 4 and 5). However, electroblot analysis confirmed the absence of disease specificity of anti-GPI antibodies for RA. Among the sera shown in Figure 4, clear anti-GPI reactivities were seen with seronegative RA (lane 1), PsA (lanes 2 and 7), and seropositive RA (lane 9).

DISCUSSION

As previously reported by other investigators (10), we detected reactivity to GPI in the serum of a number of individuals. We confirmed this reactivity to be focused on GPI itself. Yet, there were several key differences in the results from the two studies:

The strong specificity for RA patients found by Schaller et al (10) was not reproduced in our studies. We found far less difference between RA patients and nonarthritic control subjects. We found the overall frequency in RA patients to be slightly higher than that in controls, but it was by no means predominant (50 of 336 RA patients [14.8%] versus 8 of 114 controls [7.0%]; $P = 0.045$). There was some degree of variation between cohorts. The Boston cohort (16 of 55 RA patients [29.0%] versus 2 of 37 controls [5.4%]; $P = 0.01$) showed a stronger distinction between RA patients and controls than did the Winnipeg cohort (4 of 33 RA patients [12.1%] versus 7 of 31 controls [22.6%]; P not significant). Yet, in no instance did the frequency in RA patients reach the 60% value previously reported (10). Lowering the cutoff point used for positivity did not improve the discrimination between RA patients and controls (data not shown), nor did various calculated indices encompassing the reactivity to either or both forms of GPI (Table 5).

No specificity for RA compared with other arthritic diseases was observed in our study. Positive anti-GPI titers were found in other groups of patients, such as those with PsA and SpA, but, again, in only subsets of these patients. Positive titers were also found in sera from patients with Crohn's disease and patients with sarcoidosis.

Why do our results differ? Aside from the variability between cohorts discussed above, an explanation may lie in the very aggressive forms of RA sampled in the study by Schaller et al (10); a large number of those patients had Felty's syndrome. The very high prevalence of anti-GPI in the Schaller study may thus have reflected a preponderance of the Felty's syndrome form of RA. Our cohorts did not have this particular condition, but we did find a slight relationship between the aggressiveness of the arthritis and the indices of anti-GPI (Figure 3). The peculiarity of Felty's syndrome patients may be worth reexamining in future studies. Anti-GPI titers, which were highest at peaks of disease activity, may thus subside in more latent phases. However, all samples from the NIH cohort were obtained at disease onset, likely in an inflammatory setting, and the values were not significantly higher overall than those in the other groups of arthritis patients.

The pathogenic potential of the anti-GPI antibodies present in these patients is a key question. It may well be that GPI is only one of several autoantigens able to serve as a target for pathogenic antibodies. In very preliminary analyses, anti-GPI antibodies from patients with the highest titers were affinity-purified and injected into naive BALB/c mice, the strain most susceptible to arthritis transfer by K/BxN serum. In these early tests, no lasting disease was observed (Matsumoto I: unpublished observations). Yet, the interpretation of these findings is complicated by possible incompatibility between human and mouse complement and Fc receptor signaling pathways, both of which are essential to arthritogenesis (26). These results will need to be reexamined in "humanized" test systems.

In conclusion, GPI itself may prove to be the target of autoimmune attack in some patients, as it is in K/BxN mice. It is now clear that it does not provide, with the ELISA and electroblot assays now available, a useful general assay for the discrimination of RA.

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T Cell Receptor Repertoire of T Cells in the Kidneys of Patients With Lupus Nephritis

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Objective. **To determine the role of T cells in the pathogenesis of lupus nephritis (LN).**

Methods. **Renal biopsy specimens from 12 patients with systemic lupus erythematosus were used for the experiments. We analyzed T cell receptor (TCR) Vβ1–20 family genes on intrarenal T cells and on peripheral blood lymphocytes (PBLs) by nested reverse transcriptase–polymerase chain reaction (PCR) and Southern blot analysis. Nucleotide sequence was determined in the third complementarity-determining region of the TCR Vβ gene in expanded T cells. Messenger RNA (mRNA) expression levels of Th1 and Th2 cytokines on infiltrating T cells were measured by nested PCR.**

Results. **The repertoire of TCR Vβ in intrarenal T cells was relatively restricted compared with that in PBLs. The TCR Vβ8 and TCR Vβ20 genes were preferentially expressed in 6 of 12 patients (50%) and the TCR Vβ9 and TCR Vβ14 genes were expressed in 5 of 12 patients (42%). Junctional sequences of complementary DNA encoding the TCR Vβ8 and TCR Vβ20 genes in intrarenal T cells showed oligoclonal expansion, indicating antigen-driven stimulation. Interleukin-4 (IL-4) and IL-10 mRNA were highly expressed on intrarenal T cells, while interferon-γ mRNA was not detected.**

Conclusion. **Our findings suggest that T cells infiltrating the kidneys of patients with LN may recognize restricted epitopes on antigens and function as Th2-type T cells.**

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Lupus nephritis (LN) is characterized by the infiltration of mononuclear cells, mainly CD4+ T cells, and the deposition of immune complexes within the glomeruli (1). The immune deposits are caused by pathogenic anti-DNA autoantibodies and autoantigen complex. This pathogenic anti-DNA autoantibody response is dependent on CD4+ Th cells (2–6). The third complementarity-determining region (CDR3) of the T cell receptor (TCR) β chains expressed by these pathogenic Th clones bears a recurrent motif of anionic residues, suggesting it is specific for autoantigens with cationic residue.

Studies have indicated that the restricted oligoclonal T cells may play an important role in the development of various diseases, including multiple sclerosis, rheumatoid arthritis, and Sjögren's syndrome (7,8). Funauchi et al (9) reported that the levels of interleukin-2 (IL-2) and interferon-γ (IFNγ) (Th1 cytokines) were lower, while those of IL-4 and IL-10 (Th2 cytokines) were higher, in peripheral blood mononuclear cells (PBMCs) of patients with systemic lupus erythematosus (SLE) than in those of healthy subjects. Viallard et al (10) showed that IL-10 production by PBMCs was significantly higher in patients with SLE than in healthy controls, while IL-1 and IFNγ contents did not differ between SLE patients and controls. Richaud-Patin et al (11) reported high gene expression of IL-4, IL-6, IL-10, and tumor necrosis factor α in SLE patients compared with healthy subjects, while the expression of IL-1β, IL-2, and IFNγ genes was low or undetectable in PBMCs of SLE patients. These findings indicate that the preferential increase in cytokine production from Th2 cells relative to that by Th1 cells might be associated with polyclonal B cell activation seen in SLE.

In the present study, we investigated the role of T cells in the pathogenesis of LN by comparing the TCR Vβ repertoires in intrarenal T cells and peripheral blood lymphocytes (PBLs) of patients with LN, using nested reverse transcriptase–polymerase chain reaction (RT-

PCR). Our results showed that the TCR Vβ repertoire is relatively restricted in the kidneys. In addition, the junctional sequence of TCR Vβ genes from the kidney demonstrated that TCR Vβ8– and TCR Vβ20–positive T cells expanded oligoclonally and there were some conserved amino acids in the CDR3 of TCR Vβ genes. These findings suggest that limited nephritogenic antigens might activate T cells, resulting in the development of nephritis in patients with SLE.

PATIENTS AND METHODS

Patients and renal biopsies. Twelve patients with LN were referred to Tsukuba University Hospital, Toho University Sakura Hospital, and Shimoshizu National Hospital, and all met the criteria for diagnosis of SLE (12). They consisted of 2 men and 10 women, ages 17–93 years (mean 42.8), and all patients were in the active stage of SLE with LN. A percutaneous renal biopsy obtained in each patient prior to the administration of any medications and nutritional remedies showed marked mononuclear cell infiltration in the glomeruli. According to World Health Organization (WHO) criteria, 2 patients (SLEK2 and SLEK11) had class III nephritis and 10 had class IV nephritis (SLEK3–10, SLEK12, and SLEK13). Typing of HLA-DR and HLA-DQ alleles was performed by PCR combined with dot-blot hybridization using sequence-specific oligonucleotide probes, based on the protocol of the Eleventh Histocompatibility Workshop (13). The study protocol was approved by the Human Ethics Review Committee of Tsukuba University, and a signed consent form was obtained from each patient.

Histopathologic and immunohistochemical analyses. Tissue samples from the kidneys of patients with LN were fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. For immunostaining, a portion of a sample was snap frozen, and cryostat sections were cut and stained with anti-CD3, anti-CD20, anti-CD4, or anti-CD8 monoclonal antibodies (mAb; Becton Dickinson, Mountain View, CA). Cryostat sections were incubated with biotinylated rabbit anti-mouse Ig (Dako, Glostrup, Denmark), then with StreptABComplex/horseradish peroxidase (Dako), and finally with a peroxidase substrate.

RNA preparation and analysis of TCR Vβ gene use by PCR. Renal biopsy samples and PBLs of patients with LN were lysed for 10 minutes at room temperature in 20 μl of lysis buffer (40 m*M* Tris HCl [pH 8.5], 60 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol [DTT], 0.5% Nonidet P40 [NP40], and 0.05 units/μl of RNasin [Promega, Madison, WI]), and total RNA was prepared. Total RNA was reverse-transcribed using 8 μl of 5× buffer (100 m*M* Tris HCl [pH 8.5], 150 m*M* KCl, 7.5 m*M* $MgCl_2$, 25 m*M* DTT, and 0.5 mg/ml bovine serum albumin [nuclease-free; Wako, Osaka, Japan]), 2 μl of oligo$(dT)_{15}$ (50 pmoles/μl), 2 μl of dNTPs (2 m*M*) (Gibco BRL Life Technologies, Gaithersburg, MD), 0.3 μl Moloney murine leukemia virus reverse transcriptase (60 units) (Gibco BRL), and diethyl pyrocarbonate–distilled deionized H_2O in a total volume of 40 μl. The reaction was carried out at 37°C for 1 hour. The reaction mixture encoding complementary DNA (cDNA) was used for first-round PCR analysis. PCR was conducted using the method described by Sumida et al (7).

Amplification was briefly performed with *Taq* polymerase in 50 μl of standard buffer using 10 μl of cDNA with 50 pmoles each of 20 different TCR Vβ primers and Cβ primer. The sequences of the TCR Vβ and Cβ primers were obtained from previously published data (7). Oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems, Foster City, CA). Denaturing was performed at 94°C for 1.5 minutes, annealing was performed at 60°C for 1 minute, and extension was performed at 72°C for 1 minute, for 30 cycles on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Aliquots (2 μl) of the first-round PCR products were used for second-round PCR, carried out using nested 5′ primers specific for 20 different TCR Vβ family genes and a 3′ primer for the TCR Cβ gene. One-fifth of the second-round PCR product was subjected to 2% agarose gel electrophoresis and visualized by ethidium bromide staining, and then confirmed by Southern blot analysis using a digoxigenin-labeled PCR product encoding the TCR Cβ gene as described previously (14).

Cloning and junctional sequencing of TCR Vβ genes. PCR products encoding the TCR Vβ8 and TCR Vβ20 genes were ligated to plasmids using the TA cloning kit (Invitrogen, Carlsbad, CA), transformed into competent INVαF′ *Escherichia coli* cells, and grown under appropriate conditions. After selection of TCR Cβ–positive colonies, plasmid DNA was purified by alkaline lysis for DNA sequencing. Nucleotide sequences were analyzed with an ABI 377 automated sequencer (Applied Biosystems).

Expression of cytokine messenger RNA (mRNA). Complementary DNA from the kidneys and PBLs of SLE patients with LN was used for PCR, with primers specific for IFNγ (forward primer 5′-TGTTACTGCCAGGACCCATAT-3′ and reverse primer 5′-TCAGCTTTTCGAAGTCATCTC-3′), IL-4 (forward primer 5′-CTTCCCCCTCTGTTCTTCCT-3′ and reverse primer 5′-TTCCTGTCGAGCCGTTTCAG-3′), and IL-10 (forward primer 5′-ATCAAGGCGCATGTGAACTC-3′ and reverse primer 5′-AGAGCGCCAGATCCGATTTT-3′). Amplification was performed at 94°C for 1.5 minutes, 55°C for 1 minute, and 72°C for 1 minute, for 30 cycles. The PCR products were electrophoresed in 1% agarose gels containing 0.5 mg/ml ethidium bromide. The expression of these cytokines was confirmed by Southern blot analysis (IFNγ 194 bp, IL-4 317 bp, IL-10 291 bp).

Statistical analysis. Data were analyzed by one-way analysis of variance and post hoc analysis calculated by Fisher's protected least significance difference method. *P* values less than 0.05 were considered significant.

RESULTS

Infiltration of CD4+ T cells into the kidney. Histopathologic examination of the kidneys of patients with LN showed marked infiltration of mononuclear cells within the glomeruli (Figure 1A). Immunohistochemical studies using mAb against CD3, CD20, CD4, and CD8 demonstrated that the infiltrating cells were mainly CD3+ T cells and, of those, the majority were CD4+ T cells and CD8+ T cells (Figure 1B).

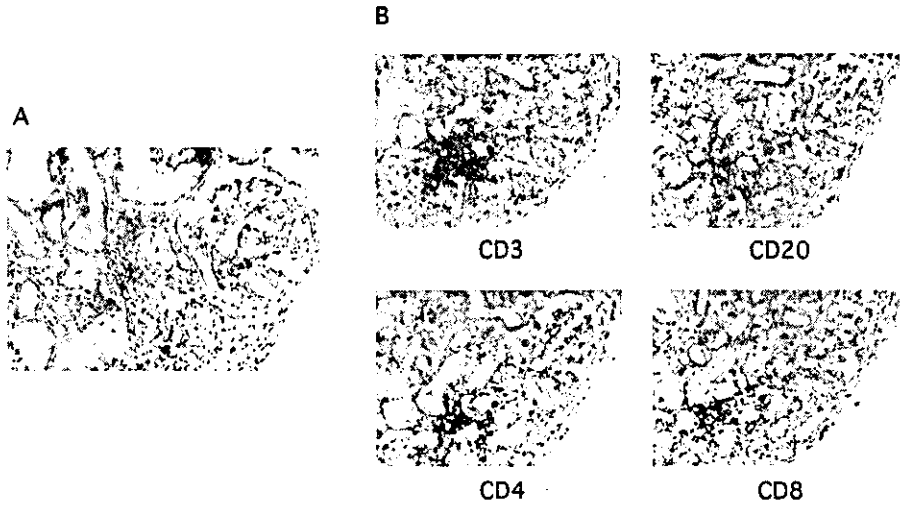

Figure 1. Infiltration of CD4+ T cells into the kidneys of patients with lupus nephritis. **A,** Histopathologic examination (hematoxylin and eosin staining), showing infiltration of mononuclear cells within the glomeruli. **B,** Immunohistochemical studies using monoclonal antibodies against CD3, CD20, CD4, and CD8, indicating that most of the infiltrating cells were CD4+ T cells. (Original magnification, × 200).

Restricted repertoire of TCR Vβ gene on intrarenal T cells. To analyze the mechanism of LN in patients with SLE, the TCR repertoire of intrarenal T cells in kidney samples was examined by PCR and Southern blot analysis. Renal biopsy specimens from 12 patients with LN were used for analysis. As controls, we used PBL samples from the same individuals. As shown in Figure 2, 1–8 TCR Vβ genes were detected in the kidneys of patients. The TCR Vβ8 and TCR Vβ20 genes were each preferentially expressed in 6 of 12 patients (SLEK4, SLEK5, SLEK9, SLEK11–13; and SLEK2, SLEK7, SLEK8, SLEK11–13, respectively) ($P < 0.05$).

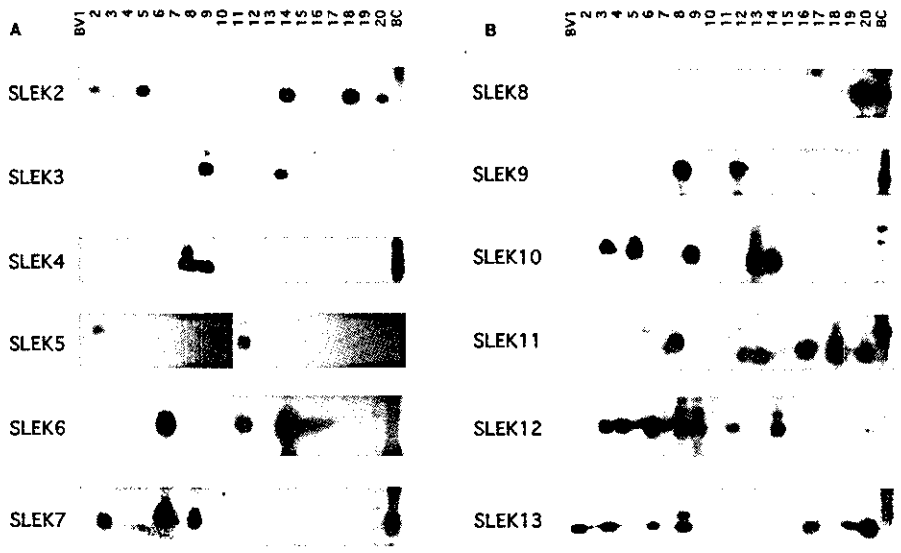

Figure 2. Restricted use of the T cell receptor (TCR) Vβ gene on intrarenal T cells of patients with lupus nephritis (LN). Renal biopsy specimens (**A**) and peripheral blood lymphocytes (**B**) from 12 patients with LN (SLEK2–13) were used for polymerase chain reaction (PCR). The TCR Vβ repertoire was examined by family PCR Southern blot analysis.

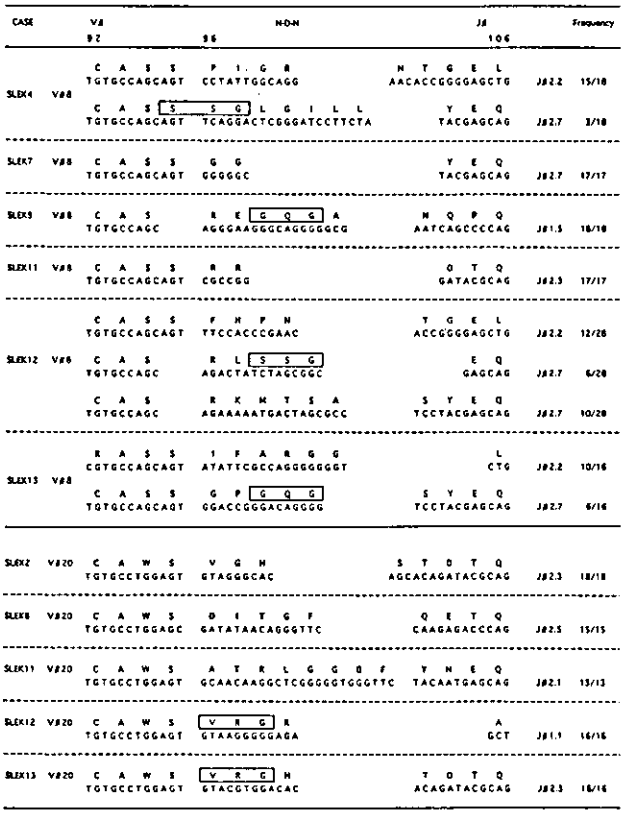

Figure 3. Junctional sequences of TCR Vβ gene in T cells from kidneys of patients with LN. The single-letter amino acid codes of the 3′ position of TCR Vβ, third complementarity-determining region, and the 5′ position of the J region are given. The conserved sequences are boxed. Frequency is the number of positive clones divided by the total number of clones. See Figure 2 for definitions.

The TCR Vβ9 and TCR Vβ14 genes were each expressed in 5 of 12 patients (SLEK3–5, SLEK10, and SLEK12; and SLEK2, SLEK3, SLEK6, SLEK10, and SLEK12, respectively). In contrast, all TCR Vβ genes were detected in PBL, and there was no predominant expression of particular TCR Vβ genes, indicating a heterogeneous TCR Vβ repertoire. These results suggest that the repertoire of the TCR Vβ genes on T cells that infiltrate the kidneys of patients with LN is restricted compared with PBLs.

Junctional sequences of TCR Vβ genes on intrarenal T cells. To examine the amino acid sequences of the TCR Vβ region, cDNA clones encoding the TCR Vβ8 and TCR Vβ20 genes from T cells infiltrating the kidneys of 6 patients (SLEK4, SLEK7, SLEK9, SLEK11–13) and 5 patients (SLEK2, SLEK8, SLEK11–

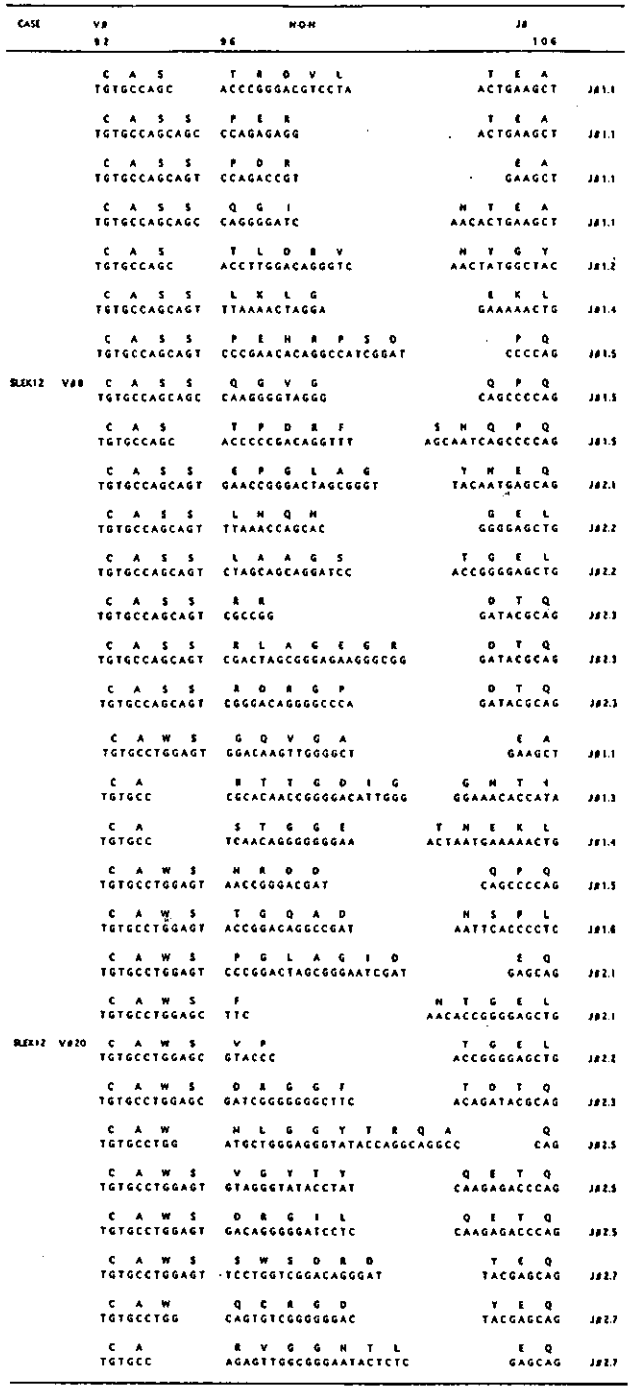

Figure 4. Junctional sequences of TCR Vβ gene in T cells from peripheral blood lymphocytes (PBLs) of a patient with LN. Representative sequences of TCR Vβ8 and TCR Vβ20 in T cells from PBLs of patient SLEK12. The single-letter amino acid codes of the 3′ position of TCR Vβ, CDR3, and the 5′ position of the J region are given. The frequency of each clone is the same. See Figure 2 for other definitions.

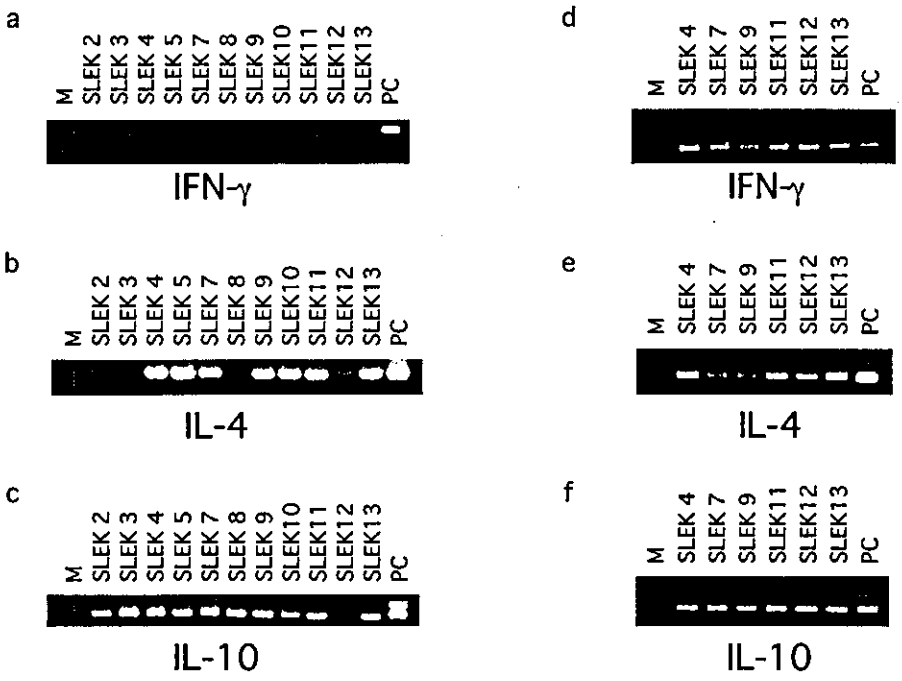

Figure 5. Expression of cytokine genes in patients with lupus nephritis. Ethidium bromide staining. **a**, **b**, and **c**, Interferon-γ (IFNγ), interleukin-4 (IL-4), and IL-10 mRNA expression, respectively, in kidneys from systemic lupus erythematosus (SLE) patients. **d**, **e**, and **f**, IFNγ, IL-4, and IL-10 mRNA expression, respectively, in peripheral blood lymphocytes from SLE patients. M = molecular size marker; PC = positive control cDNA clone.

13) respectively, were cloned and sequenced. As seen in Figure 3, junctional sequences of cDNA encoding the TCR Vβ8 and TCR Vβ20 genes on infiltrating T cells showed that these cells expanded oligoclonally, indicating an antigen-driven stimulation. The CDR3 of the infiltrating T cell clones contained conserved amino acid motifs. The SSG motif was found in TCR Vβ8 from patients SLEK4 and SLEK12; the GQG motif was found in TCR Vβ8 from patients SLEK9 and SLEK13; and the VRG motif was found in TCR Vβ20 from patients SLEK12 and SLEK13. In contrast, no conserved amino acids were detected in the CDR3 of TCR Vβ8 and TCR Vβ20 genes in PBLs from the same patient (SLEK12). The frequencies of SSG, GQG, and VRG in the CDR3 of TCR Vβ8 and TCR Vβ20 from kidneys of SLE patients were significantly high ($P < 0.05$). These results suggest that intrarenal T cells might recognize the epitopes of an autoantigen in the kidney.

Junctional sequences of TCR Vβ genes from T cells infiltrating into peripheral blood T cells. To compare intrarenal and peripheral TCR gene usage, TCR Vβ8 and TCR Vβ20 genes were examined in peripheral blood- and kidney-derived T cells from 6 individual patients with LN. Figure 4 shows data from a representative patient. There was heterogeneous TCR gene usage in the peripheral blood of this individual, with 15 distinct sequences recovered. In contrast, infiltrating T cells from the same patient showed marked clonal restriction, with only 3 sequences recovered from 28 clones. None of the latter sequences matched sequences identified from peripheral blood cDNA. These findings are consistent with the notion that antigen-driven clonal expansion results in restricted heterogeneity of the intrarenal T cells.

Expression of cytokine mRNA on intrarenal T cells. To examine the function of T cells in 11 kidneys (SLEK2–5, SLEK7–13), the expression levels of IFNγ, IL-4, and IL-10 mRNA were analyzed by the RT-PCR method. Levels of expression of mRNA for these cytokines in 6 PBL samples (SLEK4, SLEK7, SLEK9, SLEK11–13) were analyzed as controls. Intrarenal T cells showed a low expression level of IFNγ mRNA and high expression levels of IL-4 and IL-10 mRNA (Figure 5). In contrast, T cells in peripheral blood showed high

expression levels of IFNγ mRNA as well as IL-4 and IL-10. These results suggested that T cells in the kidney are Th2-type cells.

DISCUSSION

Our results show a restricted repertoire of TCR Vβ genes (Vβ8, 9, 14, and 20) in intrarenal T cells but a diverse and heterogeneous repertoire of TCR Vβ in PBLs of patients with SLE, as analyzed by the family PCR method. Recently, Massengill et al (15) demonstrated a bias in TCR Vβ gene use in infiltrating T cells in children with recent-onset LN. They examined TCR gene use on T cells in kidneys from 4 children and showed the expression of different TCR Vβ gene families. The predominant T cells were TCR Vβ18, TCR Vβ2 and TCR Vβ6, TCR Vβ17 and TCR Vβ22, and TCR Vβ6, TCR Vβ9, and TCR Vβ10 in the 4 children. The results of junctional sequence analysis described in their report demonstrated the presence of oligoclonal expansion of TCR Vβ families. Thus, our findings are consistent with those of the above study.

The sequences of the junctional regions of TCR Vβ8 and TCR Vβ20 analyzed in our study demonstrate the presence of oligoclonal expansion of TCR Vβ families, indicating antigen-driven stimulation. Moreover, there were some conserved amino acids in the CDR3 of clonally expanded T cells. These findings suggest that T cells infiltrating the kidneys of LN patients may recognize limited epitopes of antigens. What are the autoantigens recognized by T cells in the kidneys? Although there are no reports on the antigens for T cells infiltrating the kidney in SLE patients with LN, kidney-specific antigens may be the candidate antigens. Mostoslavsky et al (16) reported that anti-DNA autoantibodies cross-react with glomerular structural proteins, the acidic actin-binding protein, and α-actinin, suggesting that kidney dysfunction in SLE may be enhanced by protein–nucleic acid antigenic mimicry. It is possible that intrarenal T cells in LN recognize glomerular structural proteins, such as protein–nucleic acid, presented on the HLA–DR molecule after tissue destruction through antigen–antibody interaction. On the other hand, there is no direct evidence for the presence of glomerular structural protein specific for T cells in the kidney. Since LN is characterized by interstitial nephritis as well as proliferative glomerular nephritis, autoantigens, such as 3M1, a tubular basement membrane protein (17), might induce interstitial T cell infiltration in LN. Establishment of T cell lines from the local region of LN should shed some light to elucidate the antigens that induce T cell infiltration.

It has been proposed that an imbalance of Th1- and Th2-type T cells in the peripheral blood of patients with SLE is associated with the pathogenesis of the disease (9–11,18–20). Funauchi et al (9) found that cytoplasmic IL-2 and IFNγ levels were low and IL-4 and IL-10 levels were high on peripheral mononuclear cells of patients with SLE compared with those of healthy subjects. They predicted that the deviation of Th1 to Th2 cells in the periphery might be associated with polyclonal B cell activation in patients with SLE. In contrast, Akahoshi et al (19) demonstrated a strong predominance of Th1 cells in peripheral blood in lupus patients with WHO class IV nephritis. Using immunohistochemistry methods, Masutani et al (20) analyzed the expression of IFNγ and IL-4 on intrarenal T cells as well as peripheral blood from SLE patients with diffuse proliferative LN and showed a dominance of the Th1 response. They suggested that the peripheral blood Th1: Th2 ratio directly reflects the local histopathologic findings.

On the contrary, our study indicates that infiltrating T cells in the kidneys could produce Th2-type T cell cytokines such as IL-4 and IL-10. These findings suggest the following 2 possibilities. One is that Th2-type T cells in the kidneys might play a role in the generation of LN. The other is that Th2-type T cells accumulate in the kidneys to improve nephritis as regulatory T cells. The discrepancy between our results and the findings of Masutani et al on cytokine expression of intrarenal T cells might be due to the sensitivity of the methods used for detection of cytokines. The PCR method is more sensitive than immunohistochemistry; however, further examination of cytokine expression in the intrarenal T cells at the protein level as well as the mRNA level is warranted.

In murine models of SLE, such as (NZW × C57BL/6.Yaa)F_1, the constitutive expression of the IL-4 transgene by B cells completely prevents the development of lethal lupus-like glomerulonephritis (21). Overexpression of IL-4 results in modulation of the Th1-dominant autoimmune response in peripheral blood, resulting in a decrease of Th1-mediated IgG3 and IgG2a. Although there are no reports of cytokine production by intrarenal T cells in murine models of SLE, modification of Th1 to Th2 in the periphery might be useful as a therapeutic strategy in LN in the murine model.

In conclusion, the presentation of antigens in the context of HLA causes migration of T cells into the