Type I Diabetes and Multiple Sclerosis Patients Target Islet Plus Central Nervous System Autoantigens; Nonimmunized Nonobese Diabetic Mice Can Develop Autoimmune Encephalitis[1]

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Type I diabetes and multiple sclerosis (MS) are distinct autoimmune diseases where T cells target either islet or CNS self-proteins. Unexpectedly, we found that autoreactive T cells in diabetic patients, relatives with high diabetes risk, nonobese diabetic (NOD) mice, and MS patients routinely target classical islet as well as CNS autoantigens. The pathogenic potential of CNS autoreactivity was testable in NOD mice. Pertussis holotoxin, without additional Ags or adjuvants, allowed development of an NOD mouse-specific, autoimmune encephalitis with variable primary-progressive, monophasic, and relapsing-remitting courses. T cells from diabetic donors transferred CNS disease to pertussis toxin-pretreated NOD.scid mice, with accumulation of CD3/IFN-γ transcripts in the brain. Diabetes and MS appear more closely related than previously perceived. NOD mouse-specific, autoimmune encephalitis provides a new MS model to identify factors that determine alternative disease outcomes in hosts with similar autoreactive T cell repertoires. ***The Journal of Immunology,*** **2001, 166: 2831–2841.**

Type I (insulin-dependent) diabetes mellitus and multiple sclerosis (MS)[4] are chronic, tissue-selective autoimmune diseases that reflect the interactions of polygenic traits with ill defined environmental factors (1, 2). It is unknown what initiates and maintains autoimmunity to self-Ags expressed, respectively, in the pancreatic islets of Langerhans or CNS. Although the clinical pictures of autoimmune diabetes and MS are entirely disparate, there are many epidemiological parallels such as geographical and ethnic codistribution and shared environmental risk factors (1, 3, 4). Patients with type I diabetes can develop MS, and a familial association between both diseases has been suggested (5–8), but definitive data are still lacking.

The major predisposing genes for MS and diabetes map to generally different class II histocompatibility molecules that are common in the general population; their risk association emphasizes the major role of T lymphocytes in both diseases. In addition, gene products from nearly 20 predisposing genomic regions conspire toward development of autoimmunity and overt disease, with overlap among diabetes, MS, and several other autoimmune disorders (9, 10).

Diabetic autoimmunity is characterized by self-reactive B and T lymphocytes that target a set of proteins expressed in pancreatic β cells. Proinsulin (PI), IA2, GAD65 and 67, and islet cell autoantigen of 69 kDa (ICA69) are the major examples (11). These target self-Ags are not islet cell specific, and neither is diabetic autoimmunity; signs of celiac and thyroid autoimmunity are fairly common in patients (12, 13), and diabetes-prone NOD mice develop signs of thyroid and Sjögren's disease (14). Occasional islet-reactive T cells are found in almost 10% of the general population (15), but $<0.5\%$ of these subjects are likely to develop overt diabetes. Although it is uncertain what expands autoimmune T cell pools and what determines their tissue-destructive potential, access to islet target tissue has been suggested as a critical element in diabetes-prone hosts, despite availability of most relevant autoantigens in other tissues (16).

Myelin basic protein (MBP) is the classical autoantigen in MS patients, but major additional target proteins such as proteolipid protein (PLP) and others have been identified (17). T cell autoreactivity to myelin components is common in MS patients (18, 19), but CNS-reactive T cell lines can also be grown from blood samples of healthy donors (20, 21). Self-tolerance to myelin Ags is incomplete, and mammals readily develop experimental autoimmune encephalitis (EAE) following immunization with CNS Ags emulsified in CFA plus transient impairment of the blood-brain barrier (BBB) with pertussis toxin (PT) (22). However, incomplete self-tolerance to CNS Ags is without clinical consequences in the normal host, and it is unclear what drives the development of and allows intermittent CNS invasion by tissue-destructive, myelin-specific T cell pools in the MS-prone human.

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[4] Abbreviations used in this paper: MS, multiple sclerosis; AE^{NOD}, nonobese diabetic mouse-specific, autoimmune encephalitis; ICA69, islet cell autoantigen of 69 kDa; PI, proinsulin; PT, pertussis holotoxin; MBP, myelin basic protein; PLP, proteolipid protein; EAE, experimental autoimmune encephalitis; FDRs, first-degree relatives; SI, stimulation index; Hb, human hemoglobin; GUS, β-glucuronidase; BBB, blood-brain barrier.

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The classical concept of organ-selective autoimmune diseases holds that each is characterized by a requisite set of autoreactive T cells that mediate the disease-specific tissue damage in a susceptible host (23). Here we report a mutual lack of disease fidelity among autoreactive T cells from diabetes and MS patients. T cells reactive with islet autoantigens were common in MS patients, whereas myelin-reactive T cells were found in many diabetes patients and in first degree relatives (FDRs) with high risk to develop overt diabetes. To test whether the development of CNS autoreactivity was a typical part of diabetic autoimmunity, we examined diabetes-prone NOD mice. These animals were found to spontaneously generate similar CNS autoreactivity during prediabetes. Animals develop acute monophasic and delayed-onset relapsing-remitting autoimmune encephalitis following injection of PT, without any immunizations or adjuvants. Here we characterize this new model for quasi spontaneous autoimmune encephalitis. These animals offer the unique opportunity to examine the elements that determine organ-selective disease outcomes in the face of nonselective autoimmunity.

Materials and Methods

Patients

Currently untreated patients ($n = 38$) with stable or relapsing MS who visited the Toronto MS clinic (St. Michael's Hospital, Toronto, Ontario, Canada) for routine follow-up, and 34 healthy, adult volunteers provided blood samples for this study. Three study cohorts were recruited from the population-based, prospective follow-up program in diabetes kindreds from Allegheny County (PA) (24). Type I diabetes patients, newly diagnosed at the Children's Hospital of Pittsburgh ($n = 54$) were recruited with informed consent. Twenty-seven siblings to index cases, participating in the follow-up study (15), were recruited here because they had a high risk to develop overt diabetes (70%/15 yr), as determined by the presence of islet cell autoantibodies and risk-associated HLA-DQ genotypes (24). FDRs ($n = 78$) without autoantibodies were recruited as controls. Previous studies have identified a relatively low cumulative diabetes risk (~5%) in such FDRs, although two-thirds have diabetes-associated risk alleles (25).

Animal experiments

NOD/Lt, NOD.scid, SJL/J, NZB, NZB/w, BALB/c, and C57BL/6 mice were bred and housed in our vivarium, where the incidence of diabetes is 83% in NOD females <8 mo of age. NOD mouse-specific, autoimmune encephalitis (AE^{NOD}) was induced in NOD mice through i.v. injection of PT (200 ng, twice) 48 h apart. In adoptive transfer experiments, 1×10^7 pooled spleen cells from prediabetic (12 wk old), diabetic, or young (4 wk old) NOD females were injected i.v. into irradiated (650 rad) NOD males or into 8-wk-old NOD.scid mice. If not indicated otherwise, PT (200 ng i.v.) was injected on days 1 and 3 of a given experiment. Diabetes was determined by blood glucose measurements as described (26). Mice were frequently weighed and monitored daily for clinical signs of CNS disease, with at least one of two to three observers blinded to the protocol. The standard EAE grading (22) was modified because the clinical picture differed: 0, normal; 1, drowsy, ruffled fur, weight loss of at least 10%, flaccid tails; 2, 1 plus abnormal righting reflex; 3, impaired balance and/or ataxia, involuntary movements, weakness of limbs, usually asymmetric; 4, paralysis and/or persistent tremors, possible incontinence; 5, moribund or death. The observer scores were averaged. Animals with severe disease were sacrificed, and spleens were removed before perfusion with 40 ml PBS, followed by 10% buffered formalin. Tissues were prepared for light microscopic interpretation by staining with Luxol Fast Blue and hematoxylin and eosin. Slides were analyzed by a veterinary pathologist without prior knowledge of experimental regimen or clinical course. Final conclusions were blindly confirmed by three independent pathologists. Artifact-free video electroencephalogram recordings were performed in freely moving animals using a 1–5 monopolar electrode head connector, FET, preamplifiers and batteries, a signal conditioning device (Axon Instruments, Foster City, CA), A/D converter (MP100; Biopac Systems, Santa Barbara, CA), and a video/PC-PC video computer board for electroencephalogram and image data. The system was developed with untreated and genetically modified NOD and control mice, none of which showed spontaneous seizures.

BBB integrity

Evans Blue (2% w/v, 100 μl) was injected i.p. into NOD, NOD.scid, or encephalitis-prone SJL mice 7–10 wk of age. After 1 h, mice were sacrificed and perfused. Brains were washed in PBS. Dye was extracted (72 h) in formamide and measured (OD 620 nm).

T cell assays

PBMC from the study subjects were purified on Ficoll-Hypaque gradients and cultured (10^5/well) for 1 wk in protein-free Hybrimax 2897 medium (Sigma, St. Louis, MS) supplemented with human IL-2 (10 U/well) and 0.01–10 μg of a given test Ag (15). Proliferative responses ([^{3}H]thymidine incorporation) were expressed as stimulation index (SI, experimental/control cpm, background counts, x $\pm$ SD 1280 $\pm$ 210, range: 1000–2000 cpm). Positive responses were greater than the mean SI in OVA-stimulated cultures plus 4 SDs (15). This assay gave satisfactory results in a large, blinded study of diabetes families and in the first international T cell workshop of the Immunology of Diabetes Society (15, 27). The means and range of absolute background counts or of positive responses from diabetes or MS patients were not statistically different between the present and the previous study (15). The assay of murine T cell responses was similar except for the use of AIM-V serum-free medium (Life Technologies, Mississauga, Ontario, Canada), 72 h of culture, and a higher cell input (2×10^5/well). Conclusions from human and mouse experiments were similar when data were calculated as net cpm (experimental minus background cpm), but variations and confidence intervals were predictably larger.

Reagents

The diabetes-relevant test Ags have been described (15). Recombinant human baculo-ICA69 was a gift from J. Ilonen (University of Turku, Turku, Finland). *Escherichia coli*-derived human GAD65 was a gift from B. Singh (University of Western Ontario, London, Ontario, Canada). PI was a gift from Lilly (Indianapolis, IN), IA-2 (ICA512) was a gift from Bayer (Elkhart, IN). PT was a gift from Pasteur-Merrieux-Connaught Canada (Toronto, Ontario, Canada), and the enzymatically inactive PT B subunit was purchased from Calbiochem (La Jolla, CA). MBP was isolated from human or calf brain white matter as described (28). The components of human MBP were separated by CM-52 chromatography and HPLC. Unmodified MBP (component 1, MBP-c1) has a net positive charge of 21, component 8 (MBP-c8) a charge of 15, due to the natural conversion of arginine to citrulline (28). Water-soluble PLP was provided by D. Wood (University of Toronto, Toronto, Canada). OVA, BSA, human hemoglobin (Hb), cytochrome C, and actin, as well as mouse collagen IV and muscle extract were purchased. These were nontoxic in cultures costimulated with PI. Peptides: T cell epitope peptide (Tep69) from ICA69, residues 36–47 (ICA69–36: AFIKATGKKEDE), BSA150 peptide from BSA residues 150–164 (ABBOS; FKADEKKFWGKYLYE), as well ICA69–350 (EE GACLGPVA) were highly purified (>95%) and confirmed by mass spectroscopy. Ab reagents for cytokine ELISAs were purchased from BD PharMingen (San Diego, CA), and the assays followed the manufacturer's instructions. Standard template-calibrated RT-PCR (29) used the following primers: IFN-γ (449-bp amplicon): 5′-ACACTGCATCTTGGCTTTGC-3′, 5′-CGACTCCTTTTCCGCTTCCT-3′, internal: 5′-CCTTCTTCAGC AACAGCAAGGC-3′; IL-4 (422-bp amplicon): 5′-ATGG GTCTCAAC CCCCAGCTA-3′, 5′-CTACGAGTAATCCATTTGCAT-3′,internal: 5′-GT AGGGCTTCCAAGGTGCTTCGC-3′; CD3 (269-bp amplicon): 5′-TACTG GAGCAAGAATAGGAAG-3′, 5′-AGTCTGCAGTCTGT CCAG-3′, internal: 5′-GGCCAGCGGGACCTGTATTCTG-3′; β-glucuronidase (GUS) (321 bp): 5′-GTGATGTGGTCTGTGGCCAA-3′, 5′-TCTGCTCCATACTCGC TCTG-3′, internal: 5′-GCCAGTTTGAGAACTGGTA TAAGAC-3′. Mice were anesthetized and perfused with 40 ml of PBS; brain tissue was immediately lysed in Trizol (Life Technologies) and extracted, and 10 μg of total RNA was treated with DNase I (Life Technologies), reverse-transcribed, and calibrated as reported (29).

Statistics

Mann-Whitney *U* tests were used to compare numeric results. Significance was set at 5%; all *p* values were two-tailed. Fischer's exact test was used to analyze tables, with Katz' approximation to calculate relative risks.

Results

To analyze the T cell autoreactivity in diabetes and MS, we compared proliferative in vitro T cell responses to a panel of diabetes-associated and MS autoantigens in 38 currently untreated patients with stable or relapsing MS, 34 healthy controls, and 54 newly diabetic children, as well as MHC class II-matched FDRs to index

cases with a high ($n = 27$) or low risk ($n = 78$, see *Materials and Methods* for definitions) to develop overt diabetes.

MS patients have islet-reactive T cells

In our assay system, developed for the identification of disease-associated T cell autoreactivities (15), ~90% of MS patients (Fig. 1*A*), but few healthy controls (Fig. 1*B*) had T cells that recognized MBP component 1 (MBP-c1) or the citrullinated MBP-c8 (28). These T cells are thus MS associated ($p < 0.0001$ patients vs controls). Bovine MBP was recognized less frequently (20%). Nearly all subjects responded to tetanus toxoid, but not to other self-Ags (Hb, actin, cytochrome-C) or to a control nonself-protein (OVA).

In addition, MS patients frequently had T cells that target self-Ags associated with autoimmune diabetes (Fig. 1*A*). Responses to PI and IA-2 were about as common as in diabetics (Figs. 1*C* vs 2*C*), had the same magnitude as MBP responses ($p = 0.2$, Fig. 1*A*), and were rare in controls (Fig. 1*B*). T cell autoreactivity to other diabetes Ags was also common; one MS patient had no autoreactive T cells, another recognized only PI but not MBP, and only one had MBP-specific T cells but recognized none of the diabetes autoantigens tested. The presence of PI- and/or IA2-reactive T cells was significantly associated with MS ($p = 0.0002$) and thus provided an unexpected marker for MS ($p < 0.0001$).

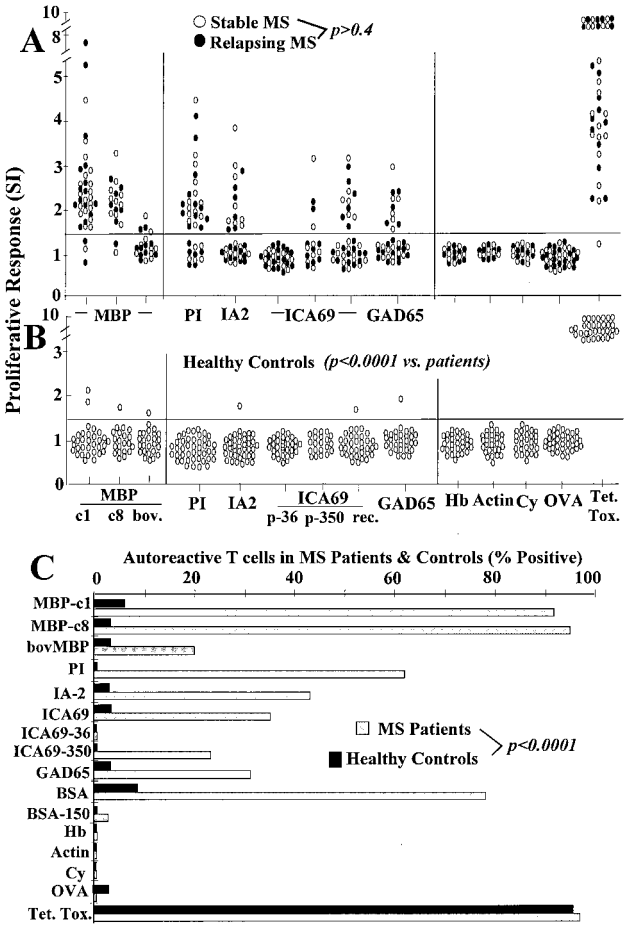

FIGURE 1. Proliferative T cell responses against self- and environmental Ags in MS patients and healthy controls. *A*, Individual response amplitudes in stable ($n = 20$, ○) and relapsing ($n = 18$, ●) MS patients are expressed as SI values. Background counts were similar in all cohorts (1000–2000 cpm). *B*, Individual response amplitudes in healthy controls ($n = 34$). *C*, Prevalence of positive responses (%) from *A* and *B*.

One-third of MS patients had ICA69-reactive T cells, but none recognized the major diabetes epitope, T cell epitope peptide (Tep69) from ICA69, residues 36–47 (ICA69–36, Fig. 1*A*) (15, 30). Four of the seventeen patients tested with the ICA69–350 peptide had positive responses, suggesting that other epitopes are targeted in MS (Fig. 1*C*). We included in these experiments BSA because this dietary protein is abnormally targeted by diabetic T cells in patients (Fig. 2*C*) and NOD mice (26). BSA (but not egg albumin, OVA) was recognized by T cells from most MS patients, but few healthy controls (Fig. 1*C*). However, MS T cells did not recognize the diabetes-associated BSA150 peptide from BSA residues 150–164 (ABBOS) (15, 31). Although disease-associated T cells target similar proteins in MS and diabetes, their epitope specificities and thus their TCRs are likely different.

T cell autoreactivity was not different in patients with stable or relapsing forms of MS (Fig. 1*A*, open vs solid circles). Thus the development of these T cell pools appears to reflect MS autoimmunity in general, rather than clinical course. The presence of islet-reactive T cells was unlikely reflective of islet destruction because no MS patient had diabetes-associated islet cell autoantibodies (ICAs and insulin, GAD65, and IA-2 autoantibodies, data not shown). In diabetes-prone subjects, such Abs accumulate in mid- to late-stage prediabetes, providing strong markers of islet destruction (32, 33). We conclude that self-reactive T cells in MS patients show no disease fidelity and commonly target CNS as well as proteins specifically associated with diabetic autoimmunity. It is unclear what triggers and sustains these T cell pools and what precludes their β cell pathogenicity in most MS patients (5, 6).

Diabetes patients have CNS-reactive T cells

A similar set of test Ags was used to analyze recently diagnosed, type I diabetic children and two cohorts of FDRs with high or low diabetes risk. Most patients had T cells targeting multiple diabetes autoantigens (a representative subset of 12 test Ags/peptides is shown here, Fig. 2) (15). Five patients (9%) had no or only one such T cell response. Hb, actin, or cytochrome C evoked little T cell response.

Twenty-nine diabetic children (53%) had proliferative responses to MBP-c1, 45% had responses to PLP (Fig. 2*A*), and 68% of those tested with both CNS Ags responded to at least one. Response amplitudes to CNS and diabetes Ags were similar ($p > 0.2$).

Multiple T cell autoreactivities are associated with high diabetes risk (15, 34, 35). Twenty-seven siblings of diabetes patients had a high disease risk (70% within 15 years, see *Materials and Methods* for definition), and most had multiple T cell autoreactivities to islet cell Ags (Fig. 2*B*). MBP and/or PLP responses were equally common in diabetics and the high risk subjects ($p > 0.3$, Fig. 2*B*), but significantly less common in low risk relatives ($n = 78$, Fig. 2*C*, $p < 0.0001$). The presence of MBP-reactive T cells predicted the presence of multiple T cell autoreactivities to islet Ags in patients ($p < 0.0001$) and high risk relatives ($p = 0.0076$, Fig. 2*D*), whereas only 6% of low risk relatives had MBP responses, and only 2% had more than one T cell autoreactivity (Fig. 2*C*). Thus the single measurement of MBP reactivity provides an unexpected marker of diabetes risk.

We concluded that diabetic patients as well as FDRs with signs of progressive prediabetes commonly generate autoimmunity to classical MS autoantigens. Thus autoimmunity in diabetes and MS targets a similar set of self-proteins, with neither disease nor tissue selectivity, although epitopes and T cell specificities appear to differ.

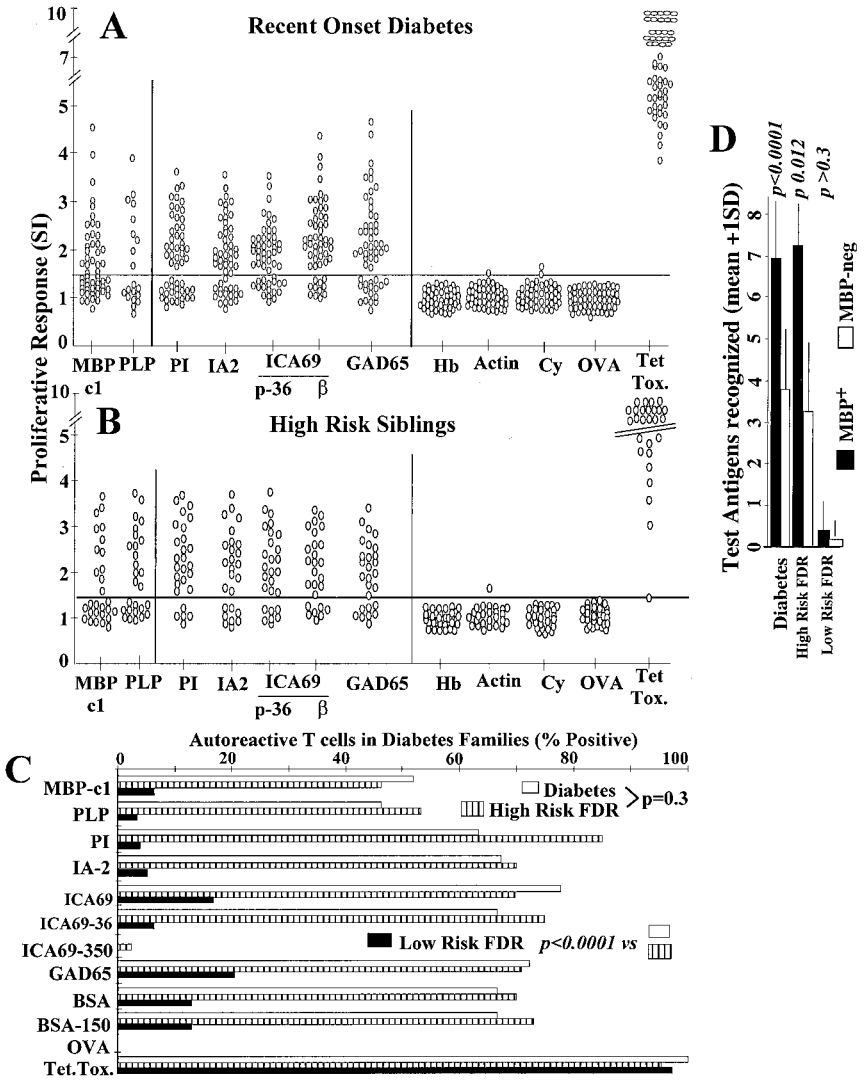

FIGURE 2. Proliferative T cell responses against self- and environmental Ags in diabetic patients and FDRs. *A*, Individual patient responses are expressed as SI ($n = 54$). Background counts were similar in all cohorts (1000–2000 cpm). *B*, Individual responses in relatives with a high risk to develop overt diabetes ($n = 27$). *C*, Prevalence of positive responses in recently diagnosed, diabetic children ($n = 54$, open row) and FDRs with high (70%, hatched row, $n = 27$) or low (~5%) diabetes risk ($n = 78$, filled row). *D*, The presence of positive MBP responses is significantly associated with multiple T cell autoreactivities in patients and high risk siblings.

Myelin-reactive T cells in NOD mice

To test the preceding conclusions and ask whether the generation of CNS autoreactivity could be part of diabetic autoimmunity in general, we examined T cell autoreactivity to CNS Ags in untreated, diabetes-prone NOD and other strains of mice of different ages (Fig. 3). CNS-reactive T cells were detected in some NOD mice by 5–7 wk of age, they were routinely observed in NOD mice older than 12 wk, and maintained in 4- to 7-mo-old diabetic animals. Thus, NOD mice spontaneously develop T cells recognizing MBP, MBP exon-2, and/or PLP, all major autoimmune targets in MS (36). As expected (37), T cell responses to diabetes-associated Ags began to appear by 5 wk of age (data not shown). There were no T cell responses against the murine self-Ags, type IV collagen, or semipurified myoglobin.

Mice from a variety of backgrounds, including C57BL/6, NZB, NZB/W, and BALB/c strains, rarely had CNS-reactive T cells detectable in our assay ($p < 0.001$ vs NOD). These observations confirmed part of our conclusion from the human data above, i.e., that CNS-specific T cells are routinely generated in the course of prediabetes; in NOD mice, this process peaks around the time of early, invasive insulitis (38).

Autoimmune encephalitis in NOD mice

We next asked whether CNS-reactive NOD T cells had pathogenic potential or whether epitopes targeted in NOD mice (39) would preclude their pathogenicity. Here we demonstrate that i.v. injection of PT (200 ng twice), without administering Freund's adjuvant or any CNS Ag, leads to the development of an age-dependent autoimmune encephalitis with a gender-independent acute disease phase and a variable, low incidence secondary disease exclusively in females, reminiscent of primary progressive and relapsing-remitting MS (Table I).

Within 1 wk after PT treatment, young adult NOD mice developed an acute disease with slow movement, ruffled fur, waddled gait, and weight loss. This acute NOD disease was age, but not gender dependent (Table I). Few mice ≤6 wk old showed any symptoms, whereas most animals >8–10 wk old developed acute, PT-induced malaise. Most of these animals recovered completely within 4–7 days. However, 12 of 78 adult females and 4 of 30 males in this series died. In addition, five females developed obvious, asymmetric paralysis and/or ataxia and were sacrificed (grade 3–4, Table I). These 21 animals with primary progressive, monophasic disease were diagnosed to have acute lymphocytic encephalitis upon necropsy (see histological examples below, Fig. 4). The diagnosis of MS-like disease was confirmed by three experienced pathologists blinded to the experimental protocols.

Of the 78 adult females, 61 appeared healthy again 2 wk after PT injection. Twelve of these (20%) relapsed 15–90 days later with variable remitting or progressive clinical courses, including one death (Table I). Four of these relapsing mice were unremarkable until 2–3 mo after the initial malaise, and then developed a

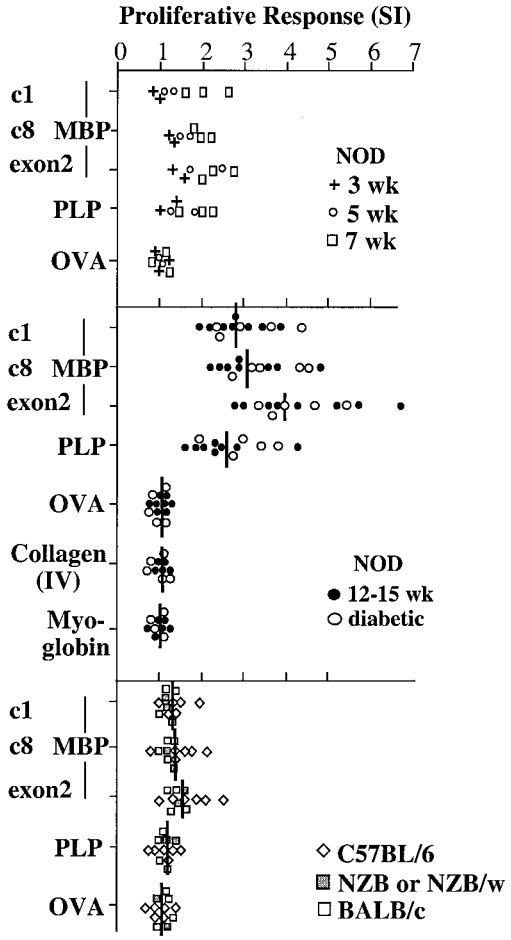

FIGURE 3. NOD mice spontaneously develop CNS-reactive T cell pools. In vitro proliferative spleen cell responses to the test Ags indicated were measured in 3- to 26-wk-old NOD and in 10- to 15-wk-old C57BL/6, BALB/c, NZB, and NZB/w mice. Data are expressed as mean SI. Horizontal bars: overall medians. Absolute background counts ranged from 900 to 1700 cpm.

progressively worsening tendency to move in tight circles, reminiscent of temporal lobe seizures (40). A typical baseline electroencephalographic (video) recording from one of these animals is shown in Fig. 4 (*top*). There was asymmetric frontal activity (Fig. 4*A*) with clear, absence-like seizure events (Fig. 4*B*). A γ-hydroxybutyrate test (41) confirmed the integrity of the thalamocortical circuitry in this animal (Fig. 4*C*). The acute and relapsing disease required facilitation by PT because we have observed no sign of this disease in over 200 untreated NOD females monitored over the past 18 mo. The incidence (Table I, $p = 0.5$) and clinical picture of acute post-PT encephalitis in adult NOD males and females was similar. However, no male developed signs of secondary relapses over 3–4 mo of follow-up. Thus the secondary relapsing-remitting NOD encephalitis follows the female gender bias of diabetes development.

NOD.scid mice were entirely free of CNS disease signs throughout 2–4 mo of observation after PT injection (Table I), even if they received 600 ng three times (data not shown). This suggested that PT-facilitated CNS disease in wild-type NOD mice depended on the presence of functional lymphocytes. Consistent with a lack of relevant observations in the literature, C57BL/6, BALB/c, and SJL mice showed no signs of disease following PT injection, suggesting that the lymphocytes required for disease had to arise in and were typical of spontaneously autoimmune NODs. The absence of clinical signs, including weight loss in NOD.scid and other control animals ruled out merely toxic effects of PT.

Histopathological analysis was performed in over 50 animals. Typical findings in brain sections from some of the 63 female and 21 male NOD mice with acute malaise 5–6 days post-PT (Table I) were characterized primarily by small, multifocal, perivascular cuffs of mononuclear cells in the cortex and brainstem (Fig. 4, *D* and *E*), and mixed inflammatory cell infiltrates in the ventricular system (Fig. 4*F*). Monophasic, acutely progressive disease, 7–10 days post-PT (Table I), was associated with more marked, multifocal, anisomorphic gliosis and mononuclear, perivascular cuffing in hippocampus, thalamus, cerebellum (Fig. 4*G*), and brain stem. Mononuclear cell infiltration was more pronounced, and often observed in the leptomeninges (Fig. 4*H*) and ventricular system (Fig. 4*I*). Multifocal plaques of vacuolar degeneration/demyelination were observed in the deep cerebellar white matter (Fig. 4*J*) and brain stem (Fig. 4*K*) of symptomatic, but not asymptomatic, PT-treated NOD mice (Fig. 4*L*).

NOD females with secondary relapses (Table I) showed lymphocytic infiltrates similar to animals with acute disease and more prominent perivascular cuffing (Fig. 4*M*). No histological changes were detected in the brains of PT-treated NOD.scid, C57BL/6, or SJL mice (Fig. 4*N*). Lymphocytic infiltration and other pathological abnormalities were rare in spinal cords from PT-treated mice (Fig. 4*O*). Collectively, these observations characterize PT-facilitated AE^{NOD} as a condition distinct from classical EAE and resemblance to human MS. In these animals (and the adoptive transfer recipients below) we did not observe evidence for frank

Table I. *Incidence and course of CNS disease in 184 mice of various strains and ages*

Strain	Age (wk)	*n*	Acute Disease				Relapsing Disease (day)			
			No disease	1–2[a]	3–4[b]	Fatal	No disease	1–2[a]	3–4[b]	Fatal
NOD	4	10	10	0	0	0	10	0	0	0
Females	6	12	9	3	0	0	12	0	0	0
	8–12	78	15	46	5[c]	12[c]	49	4[c] (d16–90)	7[c] (d15–45)	1[c] (d28)
Males	9–14	30	9	17	0	4[c]	26	0	0	0
NOD.scid	8	12	12	0	0	0	12	0	0	0
	12	14	14	0	0	0	14	0	0	0
C57BL/6	10	8	8	0	0	0	8	0	0	0
SJL/J	8	9	9	0	0	0	9	0	0	0
	12	11	11	0	0	0	11	0	0	0

[a] Grade 1–2: flaccid tail ± impaired righting reflex; ruffled fur, weight loss, slow movement.
[b] Grade 3–4: ataxia, impaired balance, asymmetric paralysis or seizures.
[c] Histopathology/RT-PCR confirmation of lymphocytic encephalitis.

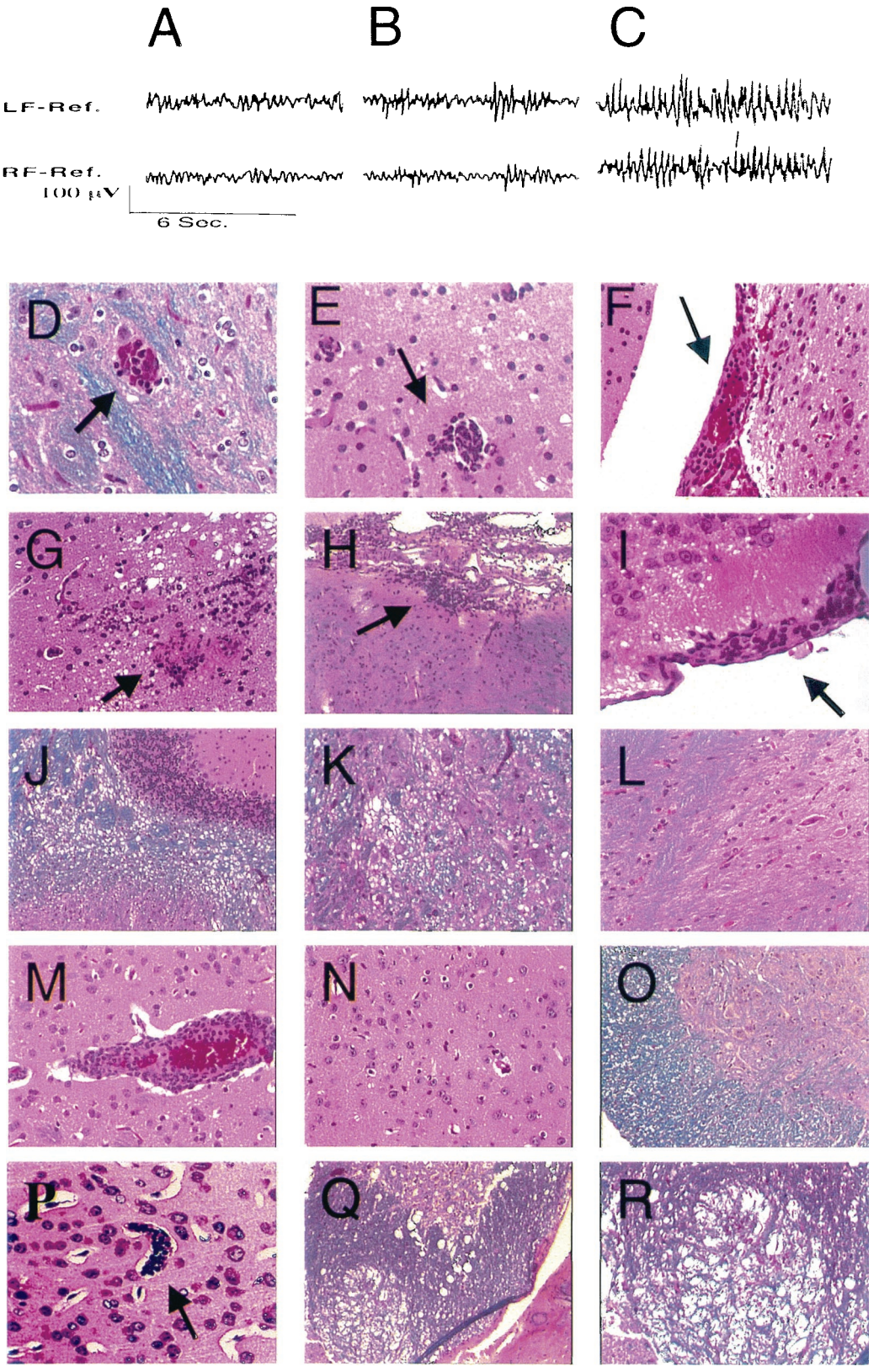

FIGURE 4. Characteristics of autoimmune encephalitis in PT-treated NOD mice. *A*, Baseline electroencephalogram recording shows lower amplitude over the right frontal region. LF, Left frontal; RF, right frontal; Ref, reference electrode located 2 mm posterior to λ and 2 mm lateral to midline. *B*, Absence-like seizures in a PT-treated NOD mouse with late relapse of neurological symptoms. Spontaneous bilaterally synchronous spike-and-wave discharges (SWD) at 6–7 cycles/s were associated with intermittent immobility, vibrissal twitching, and absence-like seizures. *C*, Subcutaneous injection of γ-butyrolactone (100 mg/kg) induced sinus wave discharges of similar amplitude and morphology within 60 min, associated with behavioral arrest and continuous absence-like seizures. *D–R*, Histopathologyy of AE^{NOD} CNS histopathology in PT-treated NOD mice with acute or chronic progressive disease. Sections were stained with Luxol Fast Blue (all original magnifications ×100 unless indicated otherwise). *D*, Shown is a transverse section of the cortex with a small mononuclear focal cuff and gliosis in a symptomatic NOD female 5 days after PT injection. *E*, One of many small perivascular cuffs in the brain stem of a

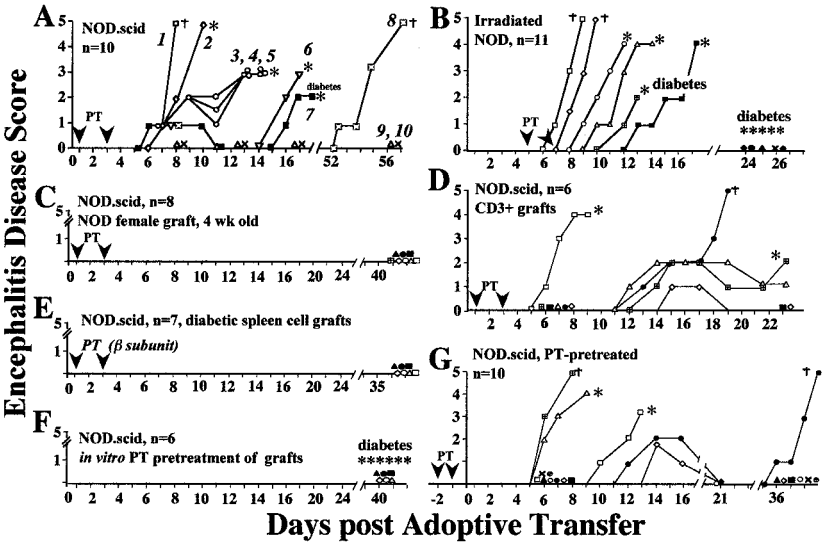

FIGURE 5. Adoptive transfer of AE^{NOD}. *A*, Spleen cells from 12-wk-old NOD females (1×10^7, numbered 1–10 were adoptively transferred into NOD.scid recipients on day 0. The timing of PT injection (200 ng, twice) is indicated by arrow heads. *B*, Diabetic spleen cells (1×10^7) were transferred to sublethally irradiated (650 rad) male NOD recipients. *C*, Spleen cells from 4-wk-old NOD females are incapable of transferring AE^{NOD}. *D*, AE^{NOD} can be induced with purified $CD3^+$ T cells from diabetic donors. Recipients had <3% detectable splenic B cells, 4–5 wk after engraftment. *E*, The B subunit of PT fails to induce AE^{NOD} in NOD.scid mice adoptively transferred with diabetic spleen cells. *F*, Diabetic spleen cells pretreated in vitro with PT for 2 days fail to transfer AE^{NOD} to NOD.scid recipients. *G*, NOD.scid mice pretreated with PT (see arrow heads) before adoptive transfer of diabetic spleen cells develop AE^{NOD}. †, Death. ∗, Sacrificed for histopathology.

autoimmunity in other tissues, but we did not perform quantitative analyses that would rule out acceleration/exacerbation of sialitis. In terms of major tissue destructive and pathogenic autoimmunity, NOD mice are prone to diabetes and AE^{NOD}.

Adoptive transfer of AE^{NOD}

Spleen cells from 12-wk-old females (10^7/recipient) were adoptively transferred into 8-wk-old NOD.scid mice (Fig. 5*A*) or diabetic spleen cells were transferred to sublethally irradiated, 8-wk-old NOD males (Fig. 5*B*). Other NOD.scid mice received spleen cell grafts from 4-wk-old NOD females (Fig. 5*C*) or highly purified splenic T cells from diabetic donors (Fig. 5*D*). Recipients received two PT injections as indicated, or two injections of the enzymatically inactive B subunit instead of holotoxin (Fig. 5*E*). In another series of experiments, adoptive grafts were pretreated for 2 days in vitro with 100 ng/ml PT before adoptive transfer into untreated NOD.scid (Fig. 5*F*), or NOD.scid recipients were pretreated with PT 2 days before adoptive transfer of diabetic splenocytes (Fig. 5*G*). For analysis of these 58 adoptive transfer recipients (Fig. 5) and 37 additional controls of adoptively transferred, but not PT-treated recipients (data not shown), we used a disease grading analogous to EAE (see *Materials and Methods*).

Following adoptive transfer of wild-type prediabetic wk 12 spleen cells into 10 NOD.scid mice (Fig. 5*A*), PT injection generated an acute disease similar to PT-treated wild-type NOD mice in all but two animals (9 and 10). Two animals progressed rapidly toward death (1 and 2), three progressed to grade 3 clinical disease over the following week and were sacrificed for histopathology (3–5), and three recovered completely (6–8). Three of the five then apparently healthy animals (6–10), developed early (6 and 7) or late (8) relapse, and two (9 and 10) remained symptom free over a 62-day follow-up period.

A comparable course of events characterized the adoptively transferred, irradiated NOD males (Fig. 5*B*). Two animals quickly developed primary progressive, monophasic disease with asymmetric neurological symptoms, ataxia, weight loss, and death. Four animals showed intermediate disease progression. All animals were sacrificed when they developed diabetes.

NOD autoimmune encephalitis is age dependent, because spleen cells from young NOD females (4 wk old) were unable to transfer disease, consistent with the absence of detectable CNS-reactive T cells at that age (Fig. 5*C*). AE^{NOD} is mediated by T lineage cells that spontaneously arise in the course of prediabetes because the disease could be transferred with purified $CD3^+$ cells from newly diabetic donors (Fig. 5*D*).

The morphology and distribution of perivascular inflammatory cell cuffs and lymphocytic meningitis in the brain were indistinguishable from those described above for PT-treated wild-type NOD mice (Fig. 4*P*). Histological examination of sections of cervical, thoracic, and lumbar spinal cord from most symptomatic, adoptively transferred NOD.scid mice were unremarkable, but in a few of these animals we observed multifocal plaques of demyelination of the dorsal, lateral, and ventral funiculi (Fig. 4, *Q* and *R*).

Thirty-seven irradiated adoptive transfer recipients of diabetic spleen cells but without PT injections were closely monitored. None developed neurological signs, malaise, or weight loss until they developed diabetes as expected, and no abnormalities in CNS histopathology were observed in the subset of mice analyzed (data not shown). A trend toward less or slower diabetes development in PT-pretreated NOD.scid mice requires further study (42).

NOD female 6 days after PT injection. *F*, Mononuclear infiltration in the periphery of the 4th ventricle over the dorsal surface of the brain stem from a NOD male with acute disease 6 days after PT injection. *G*, Infiltration and plaque of vacuolar degeneration and demyelination in the deep cerebellar white matter of a symptomatic female 7 days after PT injection. *H*, Leptomeningeal, mononuclear cell infiltration in a female with progressive symptoms 8 days after PT injection. *I*, Mononuclear infiltrations near the ventricular system of a male with severe symptoms 8 days after PT injection. *J*, Demyelination in the deep cerebellar white matter of a NOD female with acutely progressive disease 9 days after PT injection. *K*, Demyelination in the brain stem of a NOD male with severely progressive acute disease 9 days after PT injection. *L*, Unremarkable brain section from an asymptomatic PT-treated NOD female. *M*, Perivascular cuffing in the frontal cortex of a NOD female with relapsing disease 3 wk after PT treatment. *N*, Unremarkable brain section of a PT-treated, nonsymptomatic C57BL/6 mouse. *O*, Histologically normal transverse section of spinal cord from a NOD female with relapsing disease 9 wk after PT injection. *P*, One of many scattered mononuclear infiltrates in the white matter of a symptomatic, PT-treated NOD.scid mouse with severe relapse 57 days after adoptive transfer of spleen cells from a 12-wk-old NOD female. *Q*, Transverse spinal cord section with focal demyelination in a PT-treated, NOD.scid recipient of diabetic spleen cells. *R*, Magnification (×200) of *Q*.

When PT was replaced with the enzymatically inactive pertussis B subunit, no signs of CNS disease were observed (Fig. 5*E*). This suggested that AE^{NOD} required PT-mediated ADP-ribosylation of G(i) proteins (43) either in adoptive transfer recipients, in the adoptive transfer grafts, or in both. PT pretreatment of NOD.scid recipients 2 days before adoptive transfer was sufficient for AE^{NOD} development, although the engrafted cells would have little PT exposure (Fig. 5*G*). In vitro PT pretreatment of the adoptive transfer graft was insufficient for AE^{NOD} development, but these pretreated grafts were able to mediate diabetes development (Fig. 5*F*). These observation implied that AE^{NOD} requires PT targeting of a somatic NOD-.scid tissue, but not of the lymphoid and APCs in the adoptive transfer graft.

Collectively, these data demonstrate that NOD mice spontaneously generate a CNS-pathogenic T cell repertoire that can mediate a heterogeneous autoimmune encephalitis reminiscent of the clinical, histopathological, and electrophysiological spectrum of MS. Identical adoptive transfer grafts produce this heterogeneity in identical, littermate recipients, suggesting that disease course depends on stochastic elements, amenable to further study in this model. Adoptive transfer of spleen cells from diabetic animals provides a model for accelerated, high-incidence AE^{NOD}.

AE^{NOD} involves cytokine bias

We used template-calibrated RT-PCR to amplify IFN-γ, IL-4, CD3, and GUS (control) messages in brain tissue from PT-treated NOD mice (Fig. 6*A*, *lanes 1–3*) and from adoptively transferred NOD.scid mice with neurological disease (*lane 4* provides an example). Brain tissue from mice with neurological symptoms contained CD3 message, locating T cells to the CNS of symptomatic animals. Most of these mice had IFN-γ messages in the brain, whereas IL-4 transcripts were barely detectable. However, in 4 of 22 symptomatic mice tested, IL-4 transcripts were prominent, with fewer IFN-γ transcripts; one example is shown in *lane 3*. This suggested that CNS-invasive T cell pools acquire a cytokine bias, usually, but not categorically, for the Th1 cell sublineage.

CD3, IFN-γ, and IL-4 transcripts were both easily detected in spleen cell RNA from PT-treated, symptomatic (e.g., Fig. 6*A*, *lane 5*), and untreated animals (data not shown). As a control, we analyzed SJL-strain mice with MBP-induced severe EAE (Fig. *6*, *lane 6*) and found a considerable Th1 bias among spinal cord cytokine messages. Diabetic mice not treated with PT (Fig. 6*A*, *lanes 7* and *8*) but with CNS-reactive T cells in the spleen (see Fig. 3) lacked CD3 and cytokine transcripts in the brain, consistent with absence of CNS disease in untreated NOD mice.

Activation and expansion of CNS-reactive T cells during AE^{NOD}

When spleen cells from PT-treated, encephalitic mice were stimulated in vitro with CNS Ags, there was a robust, proliferative response (Fig. 6*B*, filled column), significantly higher than those in untreated, age-matched NOD mice (Fig. 6*B*, open column, $p < 0.001$). In addition, spleen cells from PT-treated, symptomatic mice showed an Ag-dependent release of IFN-γ, but little IL-4 (Fig. 6*C*). Polyclonal activation with the mitogen Con A generated balanced cytokine production. In contrast, healthy, PT-treated NOD mice (or untreated diabetic animals, data not shown) had proliferative responses to CNS Ags similar to those shown in Fig. 3, but they produced at best borderline amounts of IFN-γ and IL-4 (Fig. 6*C*).

Collectively, our data have shown that islet-specific as well as CNS-reactive T cells arise spontaneously during human and NOD prediabetes and at some point during development of CNS disease in patients with MS. In NOD mice, ignorant CNS-reactive T cells develop cytokine responses and biases during AE^{NOD}. In adoptive transfer recipients, PT seems to target G(i) proteins in host tissue rather than the graft.

Discussion

Here we report that autoreactive T cell pools in MS patients, in diabetes patients, and in high risk prediabetic relatives target a similar set of islet and CNS autoantigens. In this respect, similarities between diabetes-prone humans and NOD mice were

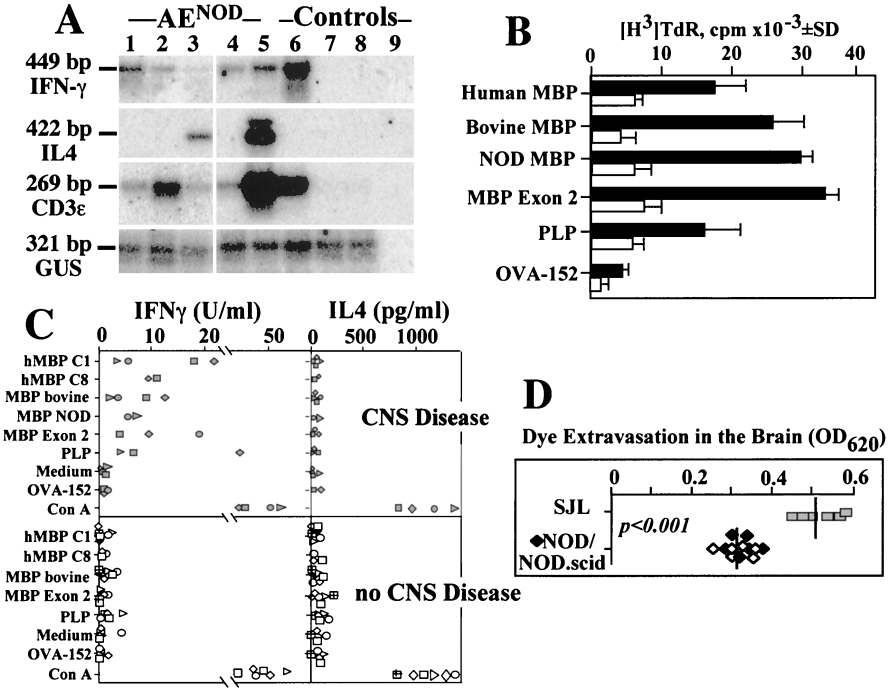

FIGURE 6. AE^{NOD} involves T cells with biased cytokine repertoire. *A*, RT-PCR and brain RNA. Southern blots of amplicons for CD3, IFN-γ, IL-4, and GUS messages. Brain RNA was obtained from PBS-perfused, PT-treated, encephalitic NOD mice (*lanes 1–3*), untreated, diabetic NOD mice (*lanes 7* and *8*), or from an adoptively transferred, symptomatic NOD.scid mouse (*lane 4*). Controls: RT-PCR of spleen cell RNA from mouse 1 (*lane 5*) or of spinal cord RNA from an SJL/J mouse with acute, MBP-induced EAE (*lane 6*); template-free "water" control (*lane 9*). *B*, Proliferative in vitro responses to CNS Ags. Pooled spleen cells from five PT-treated NOD mice with neurological symptoms (filled columns) or age-matched, untreated mice (open columns) were stimulated for 3 days as described in Fig. 3. The thymidine incorporation (mean cpm + 1 SD) is plotted. *C*, Supernatants of cultures similar to those shown in *B* were harvested after 48 h for measurements of secreted IFN-γ and IL-4 by ELISA. Con A is a polyclonal T cell mitogen. Each symbol denotes one animal. *D*, Measurement of Evans Blue-tagged albumin extravasation in the brains of SJL, NOD (solid symbols), and NOD.scid mice (open symbols) 1 h after dye injection.

remarkable. Based on these findings, we discovered a new animal model for autoimmune encephalitis, AE^{NOD}, and established its characteristics and course in broad terms. Diabetes and MS appear to be more closely related than previously perceived (5–8).

We were surprised by the lack of disease fidelity among autoreactive T cells in diabetes and MS patients. The finding that CNS autoreactivity emerged as a marker of diabetes risk in families of children with type I diabetes associated the development of these T cell pools and progressive prediabetes with strong statistical confidence. This conclusion applies to NOD mice as well, where protracted relapsing-remitting CNS disease was only observed in NOD females, prone to develop progressive prediabetes and overt disease.

The observation that PI autoreactivity showed a strong association with MS was equally unexpected, as this molecule is perhaps the most prominent marker of diabetic autoimmunity (15, 44). Although it remains obscure what initiates, expands, and sustains these T cell pools in diabetes- and MS-prone hosts, the common denominator may well be autoimmunity that breaks self-tolerance to some, but not many other proteins, of which we measured but a small subset. It should be instructive to study patients with other organ-selective autoimmune disorders and map T cell disease fidelity.

The measurement of autoreactive T cells is difficult, and we spent considerable efforts to develop and validate our serum-free assay system in diabetes families (15, 27) and in NOD mice (26, 30, 31). Although these responses have small amplitudes, they provided the impetus for NOD mouse experiments that, in turn, delineated similar CNS autoreactivities as well as functional confirmation for the presence of CNS-reactive T cells in these animals. Small proliferative T cell responses in our assay system are likely of biological relevance in unimmunized autoimmune humans and mice. We failed to detect many CNS-reactive T cells in healthy humans, unlike observations reported in many MS studies (e.g., Refs. 18 and 19). Our serum-free culture conditions appear to be insensitive for the routine detection of autoreactive T cells from normal hosts, cells that may differ functionally from those in patients (45).

Studies of NOD mice confirmed our hypothesis that CNS autoreactivity is a routine part of prediabetes. Nevertheless, we were surprised by the results because MBP was routinely targeted by NOD T cells, although it is poorly encephalitogenic in the nonautoimmune NOD cousins, IA^{g7}-bearing Biozzi mice (39). To analyze an extended range of CNS autoantigens and map NOD target epitopes should be rewarding, because we do not know whether few or many autoreactive T cell pools participate in AE^{NOD}, and whether these T cells were measured in our in vitro experiments. Autoreactivities underlying the disease now seem likely to arise spontaneously, and if so, they may be susceptible to early experimental treatments with immunotherapeutic peptides identified in such mapping studies.

The finding that PT injection at moderate doses directs progression to overt autoimmune disease in the CNS provided indirect support for the conclusions of in vitro human and mouse T cell studies, i.e., that islet and CNS self-Ags are targeted in parallel. However, it is unclear what protects the diabetes-prone host from spontaneous CNS disease, what triggers the pathogenicity of these T cells well after their initial appearance, and, by inference, what protects the islet in MS. These are not all new questions. The development of islet-reactive T cells in humans precedes disease onset by years (15). T cells of prediabetic NOD mice infiltrate islets months before disease onset (46), and disease transfer proceeds at a slow rate unless progression checkpoints are overcome, e.g., through transfer into NOD.scid (47).

The study of AE^{NOD} may shed new light on these questions. AE^{NOD} requires T cells probably arising spontaneously in young adult mice. A role for T cells was corroborated by the presence of lymphoid cells in pathological CNS lesions and results of RT-PCR studies that demonstrated CD3 and cytokine transcripts in the brain of symptomatic animals. T cells are prerequisite for diabetes and for AE^{NOD}, and both processes have critical checkpoints that protect the islet (temporarily) and the CNS (completely until PT injection). The identity of these checkpoints is unknown, but the one that governs AE^{NOD} appears to be controlled through G(i) signaling pathways, because only PT was effective in disease facilitation. This may open opportunities for studies of small molecule pharmaceuticals that modulate such pathways.

CNS-reactive T cells in prediabetic NOD mice were able to proliferate following cognate activation in vitro, but they produced very little cytokine, a property they acquired rapidly following disease onset. Our adoptive transfer experiments mapped the PT target to host tissue. Transient breaches of the BBB are thought to be an important part of the pleiotropic PT actions (48). If PT treatment promoted CNS tissue access, this would be a good candidate event associated with loss of T cell ignorance and transition to tissue-destructive autoimmunity and overt disease.

It will be interesting to determine whether the converse is true, i.e., that CNS-autoimmune NOD mice might be protected from CNS disease by a tight BBB. To begin addressing this possibility, we compared the integrity of BBBs in untreated NOD and encephalitis-prone SJL mice (Fig. 6*D*) using a standard dye extravasation assay (49). Following i.v. injection of Evan's Blue, NOD and NOD.scid mice showed significantly less dye extravasation than encephalitis-prone SJL mice.

The acute disease phase of NOD encephalitis is reminiscent of the probably TNF-α-mediated initial malaise in EAE (50). It occurs at high incidence, and its lack of gender bias separates it from the secondary relapsing-remitting disease of NOD females. This secondary, low-incidence CNS disease spans a spectrum of variable courses and outcomes. Stochastic, rather than genetic or environmental elements are likely to determine varied disease courses in littermates. Nonimmune elements may contribute to this variability, perhaps involving varied tissue access in animals receiving identical adoptive grafts. The considerable similarities between AE^{NOD} and MS imply that mechanistic studies of disease heterogeneity in NOD mice might become relevant for understanding MS heterogeneity.

Our findings contrast with the view that diabetes and MS reflect the presence of mutually exclusive T cell pools, which target tissue-selective autoantigens and, in a functional sense, would define each disease. The limited study of T cell epitopes conducted so far suggested that although the same proteins are targeted in diabetes and MS, the epitopes may differ. Mechanisms for the selection of proteins and epitopes require further study. Once somehow singled out for autoimmune targeting, these proteins may drive selection of disease-specific epitopes within these proteins, likely governed by diabetes- and MS risk-associated MHC alleles. However, it remains to be determined if and how these class II alleles affect disease outcome; NOD IA^{g7} appears fully permissive for the development of pathogenic autoimmunity in both islets and CNS.

The autoimmunity measured in MS and in diabetes patients and functionally analyzed in NOD mice was unexpectedly similar. To learn what (usually) protects diabetics from MS, and

MS patients from diabetes will be an important goal that could have therapeutic ramifications. Our observations describe a new model of quasi spontaneous, not Ag-induced autoimmune encephalitis as a platform to decipher genetic and/or environmental factors that decide on progression to diabetes or MS. Alternative roles for adhesion molecules (51), tight BBBs (52), possible abnormalities in the cbl-vav/CD28 pathways of T cell activation (53, 54), and crosses of NOD with other inbred strains (10, 55) may be among the avenues to pursue.

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