Influence of Environmental Viral Agents on Frequency and Tempo of Diabetes Mellitus in BB/Wor Rats

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Elimination of environmental viruses by cesarean derivation of the University of Massachusetts colony of BB/Wor rats increased the frequency and accelerated the tempo of spontaneous diabetes among diabetes-prone (DP) rats. In contrast, the viral-antibody–free (VAF) environment did not alter the resistance of pre-VAF diabetes-resistant (DR) rats to spontaneous and RT6$^+$ T-lymphocyte–depletion–induced diabetes. Pre-VAF and VAF rats have essentially the same lymphocyte subsets, and VAF-DP rats are susceptible to the adoptive transfer of diabetes and to the diabetes-accelerating effects of polyinosinic-polycytidylic acid injections. These results suggest that the presence of environmental viral pathogens may act to inhibit effector cell function in lymphopenic DP rats while enhancing effector cell activity in nonlymphopenic DR rats. *Diabetes* 40:259–62, 1991

The BB rat is an animal model of autoimmune insulin-dependent diabetes mellitus (IDDM) in which cell-mediated destruction of pancreatic β-cells is recognized as the pathogenetic event responsible for insulin deficiency and the ketosis-prone syndrome (1). The National Institutes of Health (NIH)-supported colony at the University of Massachusetts Medical School is comprised of diabetes-prone (DP) and diabetes-resistant (DR) family lines of inbred animals, which are designated BB/Wor rats. The similar genetic background of the inbred BB/Wor animals is particularly useful if one wishes to examine the role of environmental factors in the pathogenesis of the diabetic syndrome. In studies performed on BB rats before they were inbred, we observed that animals raised in a gnotobiotic environment were susceptible to spontaneous diabetes (2). Because DR-BB rats were not available at that time, that study did not address a possible role of environmental organisms in their resistance to diabetes. The gnotobiotic study also did not consider a more subtle question, the effect of the absence of viable microbes on the frequency and tempo of the diabetic syndrome. More recently, it was reported that lymphocytic choriomeningitis virus (LCMV) infection reduced the frequency of diabetes among DP rats, presumably by directly infecting and suppressing helper T lymphocytes (3,4). These studies did not examine the effects of LCMV on the resistance to diabetes among DR rats.

The importance of environmental organisms in the pathogenesis of BB rat diabetes is also suggested by conflicting observations about the role of RT6$^+$ T-lymphocyte depletion in the induction of diabetes among DR rats. The report that >50% of DR rats housed in conventional animal quarters (containing many pathogens) became diabetic after RT6$^+$ T-lymphocyte depletion (5) could not be confirmed when specific-pathogen–free (SPF) DR animals were used (6). To induce diabetes in RT6$^+$ T-lymphocyte–depleted SPF DR animals, a second environmental stimulus (sterile peritonitis) was required (6). The importance of the environment on the resistance to diabetes among DR rats was recently confirmed by the report that injections of the interferon (IFN)-inducing agent polyinosinic–polycytidylic acid (poly I:C) overcome the resistance of viral-antibody–free (VAF) rats to RT6$^+$ T-lymphocyte–depletion–induced diabetes and in fact induced diabetes in nondepleted DR rats (7).

During the summer and fall of 1989, the inbred SPF BB/Wor colony was cesarean derived into VAF quarters. This enabled us to study the effects of the elimination of environmental viral organisms on the incidence and tempo of spontaneous diabetes in DP and DR rats and the frequency of diabetes in DR rats after RT6$^+$ T-lymphocyte depletion. We also examined the effects of the VAF environment on the adoptive transfer of diabetes to young DP recipients and compared peripheral blood lymphocyte (PBL) subsets in

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both DP and DR rats before and after they were made VAF. Finally, we attempted to simulate viral-induced cytokine release by injecting young VAF-DP rats with poly I:C.

RESEARCH DESIGN AND METHODS

Representative litters from the DP and DR family lines of SPF (pre-VAF) BB/Wor rats (32–34 generations of sib mating) were cesarean derived into the VAF facility and fostered to VAF surrogate Long-Evans nursing females (Charles River, Raleigh, NC). From these foundation animals, we produced >9000 (34–37 generations) VAF-DP and VAF-DR rats. Animals selected to be future breeders were tested for glycosuria from 40 to 150 days of age. Diabetes was defined as 4+ glycosuria (TesTape) and blood glucose (tail blood) >14 mM (Beckman Glucose Analyzer II). Autoclaved Purina Chow 5010 (Ralston-Purina, St. Louis, MO) and acidified water were provided ad libitum, and animals were housed in polycarbonate cages containing autoclaved hardwood bedding in rooms with filtered air and a 12-h light-dark cycle. Personnel showered into the facility and wore autoclaved clothing, surgical masks, and caps.

Serum samples (10–20/mo) from adult animals were analyzed for antibodies to mycoplasma organisms and nine common murine viruses (Charles River, Wilmington, MA). Rats were examined for parasites at monthly intervals by our own personnel.

PBL were obtained from the buffy coats of EDTA-treated heart or orbital venous blood from 60- to 90-day-old DP and DR rats. Two-color flow cytometry was performed after sequential incubations with anti-CD8 (OX8) monoclonal antibody (MoAb), fluorescein isothiocyanate–conjugated goat anti-mouse IgG, biotinylated anti-CD5 (OX19) MoAb, and phycoerythrin-streptavidin. For each analysis, ~10,000 cells were analyzed for the phenotypic identification of natural killer (NK) cells ($CD8^+/CD5^-$), helper T lymphocytes ($CD8^-/CD5^+$), and cytotoxic/suppressor (C/S; $CD8^+/CD5^+$) T lymphocytes (8,9).

To determine the frequency of diabetes after $RT6^+$ T-lymphocyte depletion, 21- to 30-day-old DR rats were injected with 2 ml i.p. anti-RT6.1 MoAb containing tissue culture supernatant (TCS) 5 times/wk for 4 wk (6). To provide $RT6^+$ T-lymphocyte–depleted spleen cells for adoptive transfer, 21- to 30-day-old DR rats were treated with anti-RT6.1 TCS for 3 or 4 wk.

Adoptive transfer and poly I:C injections were performed as follows. Spleen cells from acute-diabetic VAF-DP rats (1–5 days after detection of diabetes) and $RT6^+$ T-lymphocyte–depleted VAF-DR rats were cultured with 4–5 μg/ml concanavalin A (ConA; ICN, Irvine, CA) for 3 days, and one spleen equivalent of cells was injected intravenously into 21- to 25-day-old VAF-DP rats (10). Twenty-five- to 30-day-old VAF-DP rats received 5 μg/g i.p. poly I:C (Sigma, St. Louis, MO) diluted in phosphate-buffered saline 3 times/wk for 4 wk. Cell and poly I:C recipients were tested for diabetes until 50 days of age.

One-way analysis of variance compared the means of experimental groups. χ^2-Analysis was performed on contingency tables. Calculations were performed with a Harris 1000 computer with SPSSx statistical software.

TABLE 1
Distribution of ages at onset of diabetes among previral-antibody–free diabetes-prone BB/Wor rats (generations 32–34)

	≤72 days		73–102 days		≥103 days		
Rat line	%	*n*	%	*n*	%	*n*	Total (*n*)
BA	5	9	73	122	21	35	166
BB	4	39	74	692	21	200	931
BE	3	2	79	48	18	11	61
NA	7	5	77	54	16	11	70
NB	6	9	84	122	10	15	146
PA	28	47*	61	102	10	17	166
Total	7	111	74	1140	19	289	1540

Mean ± SE age at detection of diabetes was 91.1 ± 0.3 days. Overall diabetes incidence was 70%. For explanation of rat lines, see ref. 11.
*$P < 0.01$ vs. all other family lines.

TABLE 2
Distribution of ages at onset of diabetes among viral-antibody–free (VAF) diabetes-prone BB/Wor rats (generations 34–37)

	≤72 days		73–102 days		≥103 days		
Rat line	%	*n*	%	*n*	%	*n*	Total (*n*)
BA	60	88	38	56	1	2	146
BB	27	212	65	518	8	66	796
BE	45	33	49	36	7	5	74
NA	16	18	82	90	2	2	110
NB	16	32	76	152	8	16	200
PA	50	113	48	108	3	6	227
Total	32	496	62	960	6	97	1553

Mean ± SE age at detection of diabetes was 79.8 ± 0.4 days ($P < 0.01$ vs. pre-VAF). Overall diabetes incidence was 86% ($\chi^2 = 143$, df = 1, $P < 0.001$ vs. pre-VAF). Distribution of ages was $\chi^2 = 356$, df = 2, $P < 0.001$ vs. pre-VAF. For explanation of rat lines, see ref. 11.

RESULTS

Before cesarean derivation, serological analyses indicated that the SPF BB/Wor colony was uniformly infected with Sendai and sialodacryoadenitis (SDA) viruses, negative for *Mycoplasma pulmonis*, but intermittently infected with Kilham's rat virus (KRV), pneumonia virus of mice (PVM), and GDVII viruses. SPF (pre-VAF) animals also frequently evidenced pinworm infestation. After cesarean derivation, 117 consecutive tests for *M. pulmonis*, Sendai virus, PVM, SDA/rat corona virus, KRV, H-1, and reovirus 3 (REO-3); 41 tests for GDVII LCMV, and mouse adenovirus; and 528 tests for parasites were negative. Bacterial organisms were not evaluated in SPF or VAF rats.

Seventy percent of the 32nd–34th generations of pre-VAF–DP rats became spontaneously diabetic, with a mean age at detection of 91 days (Table 1). Seven percent were detected before 73 days of age and 19% after 103 days of age. Among the six DP family lines (11), the distributions of the ages at onset of diabetes were comparable, except for the PA line, which became diabetic at an earlier age ($P < 0.01$). Eighty-six percent of the VAF-DP rats in generations 34–37 became spontaneously diabetic ($P < 0.001$), with a mean age at detection of 79 days ($P < 0.01$) (Table 2). The distributions of the ages at onset of diabetes were earlier in

TABLE 3
Peripheral blood lymphocyte subsets in previral-antibody–free (VAF) and VAF BB/Wor rats

Rat line	Natural killer cells (%)		Helper/inducer T lymphocytes (%)		Cytotoxic/suppressor T lymphocytes (%)	
	Pre-VAF	VAF	Pre-VAF	VAF	Pre-VAF	VAF
BA	6.7 ± 0.8	12.1 ± 1.3*	4.4 ± 0.5	4.6 ± 0.9	0.6 ± 0.1	0.8 ± 0.1
BB	9.0 ± 1.5	11.5 ± 1.5	5.6 ± 0.4	7.9 ± 0.9†	0.7 ± 0.1	0.9 ± 0.1
BE	12.6 ± 1.9	11.4 ± 1.8	7.0 ± 0.9	10.3 ± 1.3	0.9 ± 0.1	0.9 ± 0.1
NA	12.4 ± 2.1	10.2 ± 2.0	8.4 ± 0.9	10.2 ± 1.1	0.7 ± 0.1	0.8 ± 0.1
NB	7.7 ± 1.2	9.0 ± 0.5	7.5 ± 0.6	9.6 ± 0.7	0.6 ± 0.1	1.0 ± 0.1†
PA	7.2 ± 0.7	9.1 ± 1.4	6.7 ± 0.6	7.4 ± 0.6	0.9 ± 0.1	1.5 ± 0.3
WA‡	2.5 ± 0.3	3.3 ± 0.5	43.9 ± 1.7	45.2 ± 1.4	20.9 ± 1.2	18.5 ± 0.6

Values are means ± SE. n = 9–10 for pre-VAF, and n = 4–9 for VAF rats. For explanation of rat lines, see ref. 11.
*$P < 0.01$, VAF vs. pre-VAF.
†$P < 0.05$, VAF vs. pre-VAF.
‡Diabetes-resistant family line.

VAF animals, with 32% detected before 73 days of age and 6% after 103 days of age ($P < 0.001$). None of the VAF-DR rats studied (n = 506) became diabetic. This is comparable with the 1988–1989 years of the pre-VAF colony, wherein 2 of 1,245 DR rats became spontaneously diabetic.

Data on PBLs are given in Table 3. All DP rats evidenced increased percentages of NK cells, decreased helper/inducer (H/I) T lymphocytes, and virtually no C/S T lymphocytes compared with DR (WA line; 11) rats. VAF-BA line (11) rats had increased NK cells ($P < 0.01$), BB family line rats had increased H/I T lymphocytes ($P < 0.05$), and NB line (11) rats had greater percentages of C/S T lymphocytes ($P < 0.05$) than pre-VAF animals.

None of the 21- to 30-day-old VAF-DR rats (n = 81) treated with anti-RT6.1 MoAb for 3–4 wk became diabetic. These results are comparable to those observed with pre-VAF–DR rats, wherein 6 of 230 became diabetic after RT6$^+$ T-lymphocyte depletion (6).

The frequencies of diabetes among VAF-DP recipients of ConA-stimulated spleen cells and poly I:C injections are illustrated in Table 4. A positive outcome was the detection of diabetes before 50 days of age. ConA-stimulated acute diabetic spleen cells and RT6$^+$ T-lymphocyte–depleted DR spleen cells both readily transferred diabetes to young VAF-DP recipients. These results are comparable to those previously observed with pre-VAF animals, wherein 66 of 112 (59%) and 20 of 28 (71%) DP recipients of ConA-stimulated acute diabetic spleen cells and RT6-depleted DR spleen cells, respectively, became diabetic (6,10). Poly I:C injections accelerated the onset of diabetes in <50-day-old DP-VAF rats from 6% in untreated rats to 88% after poly I:C treatment.

TABLE 4
Frequency of diabetes in <50-day-old viral-antibody–free diabetes-prone rats after injections of concanavalin A (ConA)-stimulated spleen cells or polyinosinic–polycitidylic acid (poly I:C)

	n	%
ConA group		
No injection	0 of 22	0
Acute diabetic spleen cells	20 of 27	74
RT6.1-depleted diabetes-resistant spleen cells	17 of 20	85
Poly I:C	30 of 34	88
No injection	2 of 32	6

DISCUSSION

Our studies clearly indicate that, after cesarean derivation and the elimination of antibodies to the common murine viruses, the frequency and tempo of spontaneous IDDM were significantly increased in all family lines of inbred DP-BB/Wor rats. In contrast, spontaneous diabetes has not reappeared among VAF-DR animals, and these rats also remain resistant to RT6$^+$ T-lymphocyte–depletion–induced diabetes. Our studies also indicate that phenotypic NK and T-lymphocyte subsets were not substantially changed and that young VAF-DP rats are susceptible to the adoptive transfer of diabetes. Because the changes in incidence and tempo of spontaneous diabetes were observed over the course of three generations in all six inbred family lines of DP rats, it is unlikely that there is a genetic explanation for the observations. Furthermore, because we have not changed other colony management techniques, we are left with the absence of murine pathogens as the most likely explanation for the data presented above.

It is now well established that the pathogenesis of diabetes in the BB rat is autoimmune and that both helper T lymphocytes and NK cells appear to have important roles in the mechanism of β-cell destruction (12,13). Furthermore, the early observations that many modalities of immune suppression will prevent diabetes have been buttressed by reports that injections of various cytokines (e.g., interleukins 1 and 2, IFN-α, IFN-γ) alter the frequency and tempo of the diabetes syndrome (14,15). It is therefore reasonable to predict that environmental pathogens, and probably viral pathogens, may modulate the immune system so as to exacerbate or dampen a genetically programmed process of target cell destruction. Although viruses have been implicated in the etiology of insulin-dependent diabetes mellitus, most hypotheses assume that immune destruction of the β-cells follows direct virus–β-cell interaction (infection), with resulting alteration of β-cell antigenicity. The earlier report that gnotobiotic BB rats became diabetic and our results of increased frequency and tempo of diabetes in VAF-DP animals clearly

eliminate virus infection as a prerequisite for diabetes in this animal model. How then can we rationalize the dual observations reported above: that the absence of viral pathogens is associated with enhanced susceptibility of DP rats to spontaneous diabetes and is also associated with reduced susceptibility of DR rats to the induction of diabetes? Reports that experimental LCMV infections protect against BB and NOD diabetes by directly infecting and downregulating a subset of helper T lymphocytes suggest the possibility that environmental viruses may have infected and downregulated effector cell function in pre-VAF–DP rats while infecting suppressor cells with resulting upregulation of effector cells in pre-VAF–DR rats. It is also possible that DP and DR immune and accessory cells vary in their response to viruses and inflammatory mediators, so that the signals that result from virus and/or cytokine-receptor interaction may either enhance or inhibit the process of target cell destruction, depending on the overall immunologic milieu of the host.

These results also confirm the report that injections of IFN-inducing poly I:C accelerated diabetes in DP rats (16). Because poly I:C also induces diabetes in DR rats (7), the action of this virus-induced cytokine is not consistent with the disparate effects of the VAF environment on DP and DR animals.

Precedent for an indirect role of environmental pathogens and other immune stimuli in the pathogenesis of β-cell destruction can be derived from reports that NOD mice raised in a gnotobiotic environment have an increased frequency of diabetes (17) and that exposure of NOD mice to encephalomyocarditis D virus protects against diabetes (18). Because we have not collected data concerning the presence of bacterial organisms in our colony before and after cesarean derivation, we cannot rule out the possibility of a bacterial role in the altered frequency and tempo of diabetes in BB/Wor rats. However, the observations that correctly timed injections of complete Freund's adjuvant (emulsified mycobacteria) reduce the frequency of diabetes in NOD (19) and DP-BB/Wor rats (20) and that bacterial superantigens provide in vitro stimulation of diabetogenic effector cells in RT6$^+$ T-lymphocyte–depleted DR spleen cells (K.E. Ellerman, A.A.L., unpublished observations) are consistent with a role for environmental bacteria. Finally, a recent abstract that reported the temporal association of the presence of viral pathogens and an outbreak of spontaneous diabetes in a conventional colony of DR rats supports our hypothesis (7). Additional studies are required to identify the environmental viruses or other pathogens that may be responsible for the data reported in this article.

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