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Research Article

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Sulfated Beef Insulin Treatment Elicits CD8$^+$ T Cells That May Abrogate Immunologic Insulin Resistance in Type I Diabetes

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Abstract

The in vitro responses of T cells from 13 insulin-nonresistant and 1 immunologically insulin-resistant (IIR) type I diabetes patients to sulfated beef insulin (SBI) were analyzed. Insulin A-loop specific CD4$^+$ T cells from these patients did not respond to SBI. After 1 yr of treatment with SBI the IIR patient's T cell and antibody responses to beef, pork, and human insulin progressed from very high to nondetectable levels. This occurred in parallel to the appearance of her insulin-specific CD8$^+$ T cells, which inhibited the response of her A-loop-specific CD4$^+$ T cells to insulin. A transient increase in her CD8$^+$ anti-insulin antibody activity coincided with a relative lack of her CD8$^+$ T cell activity. CD8$^+$ T cells that regulate T cell responsiveness to insulin are probably present but difficult to detect in most type I diabetes patients. These T cells were identified in only 2 of 13 insulin-nonresistant patients who presented with lipoatrophy and insulin allergy, respectively, and who possessed high-titered, anti-insulin antibodies. Our data demonstrate that CD8$^+$ T cells play an important role in controlling peripheral tolerance to insulin and may abrogate IIR in a diabetic patient treated with SBI.

Introduction

Patients with insulin-dependent type I diabetes are treated with either heterologous beef insulin (BI)[1] and pork insulin (PI), or homologous (semisynthetic or recombinant) human insulin (HI). Anti-insulin antibodies are detectable in most of these patients (1, 2). Insulin-specific antibodies have been detected in some patients even before the onset of the disease and insulin treatment (3, 4). This shows that BI, PI, and HI are immunogenic in humans. In fact, in 1 out of 10,000 type I diabetics, high-titered anti-insulin serum antibodies of the IgG subclass may even elicit immunologic insulin resistance (IIR) (1). Treatment of diabetics with a hormonally active yet nonimmunogenic form of insulin is therefore highly desirable. We show here that treatment of an IIR type I diabetic patient with sulfated beef insulin (SBI) caused the level of insulin required to maintain this patient to decrease dramatically within 1 yr. This occurred in parallel to a marked decrease (undetectable) in both her serum levels of anti-insulin antibodies and her in vitro T cell proliferative response to insulin, and the emergence of her insulin-reactive CD8$^+$ regulatory T cells. CD8$^+$ T cells that regulate responsiveness to insulin were also detected in 2 of 13 additional insulin-nonresistant type I diabetic patients tested. These two patients did not receive SBI treatment, possessed high titers of anti-insulin serum IgG antibodies, and their CD8$^+$ T cells were not insulin specific. These data provide further insight into the design of a bioactive, nonimmunogenic form of HI for the treatment of type I diabetes.

Methods

Patients. 1 immunologically insulin-resistant patient (L.B.) and 13 other insulin-nonresistant type I diabetic patients treated either at the Women's College Hospital Endocrinology Clinic or at Hôpital Michel Levy Endocrinology Clinic were studied. The clinical data of these patients are summarized in Table I. Studies were approved by the institutional Human Subjects Research Review Committee, and written informed consent was obtained from each patient before blood sampling. Dr. A. Kenshole (Women's College Hospital, Toronto, Ontario, Canada) and Dr. B. Vialettes (Hôpital Michel Levy, Marseille, France) were the consultant physicians for these patients.

Antigens. Monocomponent, zinc-free, crystalline PI, BI, and HI, as well as SBI, were obtained from Connaught Novo Ltd. (Willowdale, Ontario, Canada). Recombinant human insulin was generously provided by Eli Lilly Canada Inc. (Scarborough, Ontario, Canada). Insulin was reconstituted in 0.06 M HCl, pH 5, aliquotted, and lyophilized. Tetanus toxin (TT; 5,000 Lf/ml) was obtained from Calbiochem-Behring Hoechst (La Jolla, CA). Both ovalbumin (OVA) and the random terpolymer of glutamic acid^{60}, alanine^{30}, tyrosine^{10} (GAT) were purchased from Sigma Chemical Co. (St. Louis, MO).

Antibodies. Purified mouse anti-human CD8 and mouse anti-human CD4 MAbs were obtained from Ortho Pharmaceuticals Canada (Willowdale, Ontario, Canada). Culture supernatant from the MCT 4 anti-human CD8-producing mouse B hybridoma was kindly provided by Dr. Tak Mak, Ontario Cancer Institute (Toronto, Ontario, Canada), and was also used in T cell proliferation assays. In the latter assays dose titration curves of the anti-CD4 and anti-CD8 antibodies (starting concentration, 10 μg/ml) were generated to determine their effect on insulin-specific in vitro T cell proliferative responses. Mouse ascites fluid containing anti-CD4 (13.B8.2) or anti-CD8 (10.D11.5) MAbs (5) were kindly provided by Dr. D. Olive (U119, Institut Nationale de la Santé et de la Recherche Medicale, Marseille, France) and used for the cell depletion experiments described below. The 82C mouse anti-I-A^k MAb (6) was found to react with HLA class II molecules expressed by an EBV-transformed lymphoblastoid B cell line established from patient L.B.

Anti-insulin antibody response. The anti-insulin serum IgG antibody response of most of the patients studied, reported as units/milli-

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; BI, beef insulin; GAT, random terpolymer of glutamic acid^{60}, alanine^{30}, tyrosine^{10}; HI, human insulin; IAA, insulin autoantibody; IIR, immunologic insulin resistance; OVA, ovalbumin; PI, pork insulin; PPD, purified protein derivative; SBI, sulfated beef insulin; TT, tetanus toxoid.

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Table I. Clinical Data of Type I Diabetes Patients Studied

Patients	Immunologically insulin resistant	Insulin nonresistant
Number	1	13
Age (*yr*)	19	41±26
Sex (*n*)		
Female	1	9
Male		4
HLA-DR type		
DR 2/–		2
DR 3/3		1
DR 3/4	1	7
DR 4/4		3
Duration of disease (*yr*)	17	12±13
Insulin treatment		
Lente		8
Lente, Humulin + NPH		3
Lente, Humulin, SBI	1	
Humulin		2

liter, was evaluated in an RIA by Dr. W. C. Sturtridge, Protein Hormone Laboratory, Toronto General Hospital (Toronto, Ontario, Canada) as previously described (7). The lower limit of detection of this assay is 1 μU/ml of serum IgG.

Lymphocyte cultures. PBL were isolated from heparinized blood by Ficoll-Hypaque sedimentation and then separated by sheep erythrocyte (E)-rosetting into E^+ T cells and E^- B cells (and macrophages). Red blood cells in the E^+ T cell population were lysed by hypoosmotic shock and the T cells were recovered by centrifugation. Cultures were set up in wells of a round-bottom 96-well Falcon microtiter plate (Becton Dickinson, Mountain View, CA), and contained 10^5 T cells and 3×10^4 irradiated (3,000 rad from a cesium-137 source) B cells/macrophages, used as antigen-presenting cells (APC), in 150 μl of complete medium (RPMI 1640 supplemented with 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin [Flow Laboratories, Burlington, Ontario, Canada], 5×10^{-5} M 2-mercaptoethanol, and 5% heat-inactivated human A^+ serum [provided by Dr. G. Sinclair, Canadian Red Cross, Calgary, Alberta, Canada]). Either insulin (BI, PI, SBI, HI) GAT, TT, or OVA was added at various concentrations to the cultures. On day 7 of culture, 1 μCi of tritiated thymidine ([^{3}H]TdR; Amersham Canada Ltd., Oakville, Ontario, Canada) was added and the plates were harvested on day 8 using a Titertek Harvester (Skatron Inc., Sterling, VA). [^{3}H]TdR incorporation was measured in an LKB-Wallace 1217 RACK-BETA scintillation counter (LKB Instruments, Inc., Gaithersburg, MD). Data are expressed as the mean counts per minute of triplicate cultures±SEM. In the case of patient L.B., who was tested on several occasions, ratios of response were calculated by dividing mean counts per minute in triplicate cultures at antigen concentrations that gave maximal stimulation by background (no antigen) mean counts per minute in triplicate cultures. Mean ratios of < 2 were indicative of a negative response. Responses of > 2 SD above the mean of the negative response were considered to be positive responses. Statistical comparisons were performed with the use of the *t* test. Other patients were tested on two or three separate occasions, and their responses that did not differ significantly on these occasions were subjected to similar statistical analyses.

For secondary in vitro T cell proliferation assays, primary cultures of E^+ T cells were set up and contained 3×10^4 irradiated (3,000 rad) APC, 250 μg/ml insulin, and, when indicated, 0.5 μg/ml of either the anti-CD4 or anti-CD8 MAb. Cells were harvested on day 10 of culture. Viable cells were recovered and replated with freshly isolated APC. After a further 4 d of culture [^{3}H]TdR was added and [^{3}H]TdR incorporation was quantitated.

Cell panning. Petri dishes (100 × 15 mm; Falcon Plastics, Cockeysville, MD) were coated for 1 h (8) at 20°C with 10 μg IgG/ml of purified anti-CD8 (Ortho Pharmaceutical, Raritan, NJ) in 0.05 M Tris buffer, pH 9.5. The plates were then washed three times with PBS containing 1% FCS. E^+ cells were resuspended at 10^7 cells/ml in 3 ml of PBS–5% FCS and incubated at 4°C for 90 min. After 45 min unattached cells were redistributed by tilting and swirling the plate. After an additional 45 min the nonadherent cells were removed by swirling, decanting, and washing the plate twice. To recover the adherent cell population the plate was filled with 10 ml PBS–5% FCS and the surface was flushed with a Pasteur pipette. The relative enrichment of the adherent cells was checked by flow cytometry. Cells were incubated with either purified anti-CD4 or anti-CD8 for 30 min at 4°C, washed, and then further treated for 30 min at 4°C with an FITC-conjugated $F(ab')_2$ of goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). After washing, the stained cells were enumerated in a FACS (EPICS V; Coulter Electronics Inc., Hialeah, FL). $CD4^+$ and $CD8^+$ T cells were each enriched to ~ 85–90% purity.

Cell depletion. Cells were incubated (10^7/ml) with a 1:100 dilution of ascites containing either an anti-CD4 (13.B8.2) or anti-CD8 (10.D11.5) MAb for 30 min at 4°C and then washed. Cells were mixed with goat anti-mouse Ig-coated magnetic beads (0.1 ml beads/10^7 cells; Dynal, Inc., Great Neck, NY) for 30 min, and bead-adherent cells were subsequently removed with a magnet. Cell recovery was higher than in panning experiments. Purity of $CD4^+$ and $CD8^+$ subsets was established by flow cytometry on an ODAM ATC 3000 flow cytometer using an FITC-coupled rabbit anti-mouse IgG (Silenus, Eurobio, France) as a second step reagent. Generally, depleted cells were found to be > 95% enriched for the $CD4^-$ or $CD8^-$ phenotypes, respectively, and were used as such in experiments.

Results

Immunologic insulin-resistant type I diabetic patient. Patient L.B. was an HLA-DR 3/4 (Dw13, DQw2/w3) 21-yr-old female who developed type I diabetes at the age of 2. Her parents and brother (age 17; HLA identical to L.B.) are currently nondiabetic. However, her mother and her mother's identical twin sister both had hypo- and hyperthyroid dysfunction. At the end of 1982, after several years of insulin treatment, L.B. presented with acute insulin resistance. She was minimally over ideal body weight and showed a marked increase in insulin requirements without evidence of accompanying acanthosis nigricans or any endocrinopathy or infection. From 1983 to 1987 we monitored her immunological and endocrinological status and grouped these analyses into four different time periods (Fig. 1) according to her levels of anti-insulin antibody production and type of insulin treatment. During the first period (January 1983–June 1984), her severe resistance to insulin was ascribed to a high titer (15,000 μU/ml) of anti-insulin antibodies (Fig. 1 *A*). At the time, her maintenance dose of 130 U/d of Lente regular insulin (BI plus PI) was excessively high (Fig. 1 *C*), and her level of glycosylated hemoglobin (19.6%) was markedly elevated (Fig. 1 *D*). L.B. was treated with steroid (10 mg prednisone/d) for 10 d, after which her antibody titer decreased to 7,200 μU/ml. She was subsequently maintained on 85 U/d of Lente insulin until May 1983 when her antibody titer rose to 16,300 μU/ml. She was then treated successively during 1 1/2 yr with purified PI (77 and 105 μU/d) and Humulin (recombinant HI; 95 U/d) with no evidence of reduction of her anti-insulin antibody titer. In fact, her antibody titer reached a peak of 47,000 μU/ml in June 1984 while she was still being treated with Humulin (Fig. 1 *A*). Accordingly, her treatment was changed to SBI (150 U/d) in June 1984 (Fig.

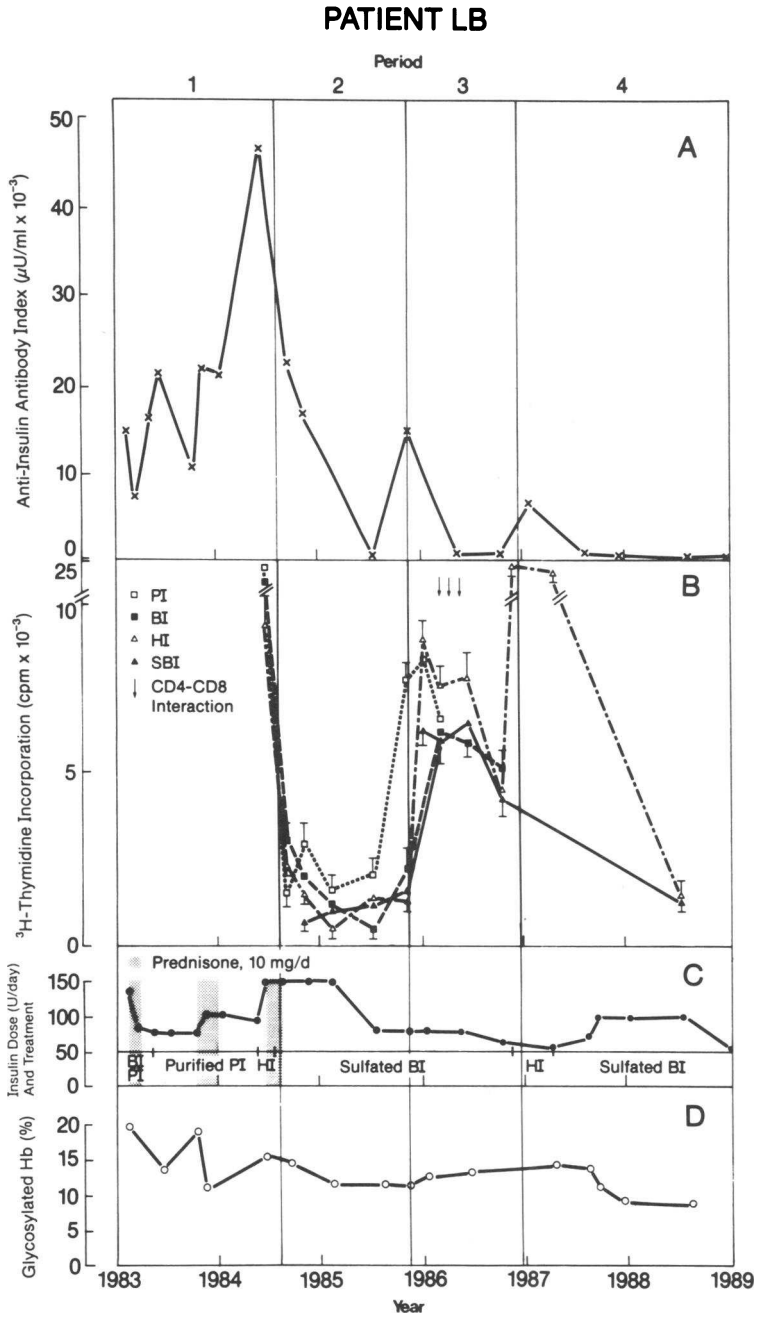

Figure 1. T and B cell reactivity to insulin in an immunologically insulin-resistant type I diabetic patient (L.B.). The immune responses to various species of insulin and other control antigens, as well as the relevant clinical data of patient L.B. during the four indicated periods (see text) between 1983 to 1989 are shown. *A*, Anti-insulin serum IgG antibody index as determined by RIA. *B*, Polyclonal T cell in vitro proliferative responses to insulin and other antigens. HI, PI, BI, and SBI were each used at 500 μg/ml. Results were quantitated by [^{3}H]TdR incorporation, and SD were < 12% of the geometric mean. The positive control responses to the GAT (used at 1 mg/ml) and TT (used at 1 U/ml) antigens ranged from 7,000 to 21,000 cpm throughout this study, with the exception that after steroid therapy at the beginning of period 2 these antigens elicited responses of ~ 3,000 cpm. No antigen control responses ranged from 500–1,500 cpm, with the exception that responses of about 4,000–6,000 cpm were noted for most (January 1986–November 1986) of period 3. The times at which the in vitro analyses of interaction between $CD4^+$ and $CD8^+$ T cells were performed are presented (*arrows*). *C*, The type and maintenance dose of insulin and duration of treatment are shown. Prednisone was administered at a dose of 10 mg/d for 10 d during the months indicated (□). *D*, Levels of glycoslyated hemoglobin (HbA_{1C}) were determined by a Corning electrophoretic procedure (28) (normal range, 5.6–7.4%).

1 *C*). As had been done in February 1983, between October 1983 and June 1984 L.B. was administered steroid (prednisone, 10 mg/d) intermittently (Fig. 1 *C*) in an attempt to decrease her anti-insulin antibody formation as different insulin (with the exception of Humulin) types were introduced.

We first tested L.B.'s T cell response to insulin in vitro during period 1 in June 1984 immediately after administration of Humulin and detected an extremely potent and crossreactive proliferative response (Fig. 1 *B*). At this time concentrations of insulin as low as 50 μg/ml stimulated significant T cell responses (9). Subsequently, it was necessary to use insulin at a concentration of 250–500 μg/ml to elicit detectable polyclonal in vitro T cell responses to this antigen as previously reported (9, 10). After short-term steroid therapy and a switch to SBI treatment (150 U/d), L.B.'s T cell response to various forms of insulin and anti-insulin antibody titer both decreased progressively and in parallel over 1 yr during period 2 (July 1984–November 1985) to nonsignificant levels in July 1985. Her diabetic condition was rather stabilized at this time since she required only a relatively low dose of SBI (80 U/d) and her glycosylated hemoglobin level in August 1985 was 9.2%. Despite a slight and transient reactivity to SBI, BI, and HI, and even greater reactivity to PI in November 1985, L.B. maintained a pattern of relatively low or no reactivity to insulin for most of 1986 during period 3 (November 1985–December 1986). As a result, on 1 November 1986 a trial treatment with Humulin was reinstituted. About 1 mo later at the end of November 1986 her T cell response to HI increased considerably (Fig. 1 *B*). During 1987 L.B. continued to display a high level of T cell reactivity to HI as noted in March 1987 (glyco-

sylated hemoglobin, 14.1%). Subsequently, her diabetic status grew progressively worse on a maintenance dose of Humulin of 67 U/d, and her treatment was changed again in July 1987 from Humulin to 70 U/d of SBI; her level of glycosylated hemoglobin in August 1987 was 11.3%. Thus, it appears that L.B. responds immunologically to the various forms of insulin given to her other than SBI. This indicates that her diabetes can be effectively treated only by the administration of SBI. L.B. was effectively maintained on 100 U/d of SBI from August 1987 until July 1988, and her dose of SBI was recently lowered to 60 U/d.

Although treatment of L.B. with SBI generally decreased both her T and B cell responsiveness to insulin, no such correlation was found for L.B.'s T cell responses to other control antigens (Fig. 1 *B*). For example, her level of T cell responsiveness to TT noted in June 1984 was reduced only by half in February 1985, while both her T cell and antibody responses to insulin decreased much more dramatically to control levels in the interim. From November 1985 to October 1986, after almost 1 yr of treatment with SBI, L.B. retained a significant T cell response to GAT and TT, while her T cell responsiveness to insulin declined to an insignificant level. From March 1986 to October 1986 the ratios of L.B.'s antigen-specific response to background response for the GAT and TT antigens were somewhat lower than those observed in 1984 and 1985. The actual level of the antigen-specific T cell responses to these antigens remained about the same as those seen in 1984 and 1985. However, the background response (i.e., autoreactive T cell proliferative response in the absence of exogenous antigen) increased ~ 5–10-fold (Fig. 1 *B*).

$CD8^+$ regulatory T cells in an immunologically insulin-resistant type I diabetic patient. Experiments were performed on three different occasions from April to October 1986 to determine whether L.B.'s lack of insulin responsiveness was mediated by $CD8^+$ regulatory T cells. The addition of anti-CD8 to L.B.'s T cells enhanced her T cell responses to SBI and HI by about four- and sixfold, respectively (Table II). No significant effect of anti-CD8 treatment on her responses to OVA, GAT, and TT was noted. In contrast, her responses to these antigens were reduced by the addition of anti-CD4. This reduction was significant only for the OVA and GAT responses, but in two other experiments (not shown) her T cell response to TT was also significantly decreased (0.2-fold response). The anti-CD8-induced enhanced response of L.B.'s T cells is specific since this treatment did not increase the responses of T cells from 13 other type I diabetic patients. Table II demonstrates that the responses of three of these patients to the various antigens tested were reduced significantly in the presence of anti-CD4 but not anti-CD8. These data suggest that $CD4^+$ and $CD8^+$ T cell subpopulations proliferate simultaneously in culture, with a predominant growth of $CD4^+$ T cells. However, in the case of LB a functionally dominant $CD8^+$ T cell subpopulation prevented the activation of a $CD4^+$ T cell subset.

This result raised the possibility that the activity of L.B.'s $CD8^+$ T cells might have increased to a detectable level after April 1986. Indeed, between January and March 1986 (beginning of period 3; see Fig. 1) we noted a transient in vitro primary T cell response to insulin (HI, PI, and BI) that could be inhibited by either anti-CD4 or anti-I-A^k (Table III, experiment 1). Treatment with anti-CD8 was without effect. When

Table II. Effect of Anti-CD8 and Anti-CD4 MAbs on Insulin-specific T Cell Responses

		Proliferative response				
			Anti-CD8*		Anti-CD4	
Patient (HLA-DR type)	Antigen‡	Control cpm × 10^{-2}	cpm × 10^{-2}	Fold-response	cpm × 10^{-2}	Fold-response
L.B. (DR 3/4)	HI	39±3	227±28	5.8	49±4	1.2
	SBI	53±5	213±15	4.0	60±6	1.1
	OVA	226±17	218±20	1.0	61±7	0.3
	GAT	125±11	200±9	1.6	52±4	0.4
	TT	99±7	161±14	1.6	79±8	0.8
	None	42±5	70±11	1.7	29±3	0.7
L.E. (DR 3/4)	HI	43±3	36±5	0.8	15±1	0.3
	TT	214±12	201±19	0.9	31±4	0.1
	None	25±4	40±6	1.6	10±2	0.4
B.S. (DR 4/4)	HI	352±19	297±30	0.8	140±16	0.4
	OVA	245±8	225±18	0.9	60±7	0.2
	TT	235±16	305±27	1.3	nt	nt
	None	50±6	80±10	1.6	20±3	0.4
G.E. (DR 3/3)	HI	161±14	157±14	1.0	28±4	0.2
	OVA	106±13	122±11	1.2	nt	nt
	None	50±2	29±4	0.6	15±3	0.3

nt = not tested. * Primary in vitro T cell proliferative responses to various antigens including HI were evaluated in cocultures of 10^5 E^+ cells and 3×10^4 autologous (irradiated 3,000 rad) E^- cells derived from four type I diabetic patients. The anti-CD8 and anti-CD4 antibodies were used at 0.5 μg/ml, which represents a concentration in the linear range (i.e., one twofold dilution below the plateau level of inhibition) of the respective antibody dose titration curves obtained for each patient. Results are expressed as the mean [^{3}H]TdR counts per minute of triplicate cultures ± SD of the mean. Similar results were obtained in three experiments. Statistically significant ($P \leq 0.05$) indices of the fold-response with respect to the no antigen control indices are underlined. ‡ HI and SBI were each used at 500 μg/ml, GAT at 1 mg/ml, TT at 1 U/ml, and OVA at 1 mg/ml.

Table III. Effect of Anti-CD4, Anti-CD8, and Anti-I-A^k MAbs on L.B.'s Insulin-specific T Cell Responses at the Beginning of Period 3

MAb	Proliferative response		
	Control	TT	PI
		cpm × 10^{-2}	
Experiment 1*			
None	47±3	290±10	268±21
Anti-CD4	nt	105±13	98±5
Anti-CD8	nt	167±4	290±31
Anti-I-A^k	nt	21±2	10±1
Experiment 2‡			
None	42±3	177±30	228±35
Anti-CD4	45±2	43±6	53±21
Anti-CD8	44±1	9±1	108±11

nt = not tested.

* In experiment 1 primary in vitro proliferative responses of L.B.'s T cells to the TT, PI, and control (none) antigens were evaluated at the beginning of period 3 (see Fig. 1) as described in Table II. Inhibition of these responses was examined after the addition of either of the anti-CD4 and anti-CD8 MAbs (each used at 0.5 mg/ml final concentration) or the anti-I-A^k 82C MAb (used at 5 mg/ml final concentration).

‡ In experiment 2 the anti-CD4 or anti-CD8 MAbs (0.5 mg/ml final concentration) were or were not (none) added to a primary culture of PI-primed L.B.'s T cells for 10 d. Viable T cells were recovered from this culture and replated with fresh APC in the presence of various antigens (TT, PI, none) and in the absence of MAbs in a secondary proliferative response that was quantitated 4 d later (see Methods).

TT was used as a positive control antigen both anti-CD4 and anti-CD8 inhibited T cell proliferation. In contrast, both anti-CD4 and anti-CD8 inhibited L.B.'s in vitro secondary T cell responses to insulin (PI, Table III, experiment 2). Similarly, these MAbs completely inhibited the secondary T cell responses to TT. Although the direct action of purified CD8$^+$ T cells was not tested in culture at this time, both the greater inhibition of a primary response noted with anti-CD4 rather than anti-CD8 and the inability of anti-CD8 to enhance T cell responsiveness to insulin suggest that CD8$^+$ regulatory T cell activity was insignificant at the beginning of period 3. This relative lack of CD8$^+$ T cell activity also coincides with the transient increase in anti-insulin antibody activity noted during this time (Fig. 1 *A*).

To further investigate the function of L.B.'s CD8$^+$ T cells, we assayed the activity of her CD4$^+$ and CD8$^+$ T cells enriched (~ 85–90% pure) by panning. The responses of her CD4$^+$ T cells to HI and SBI were enhanced about twofold by anti-CD8 treatment (Fig. 2), whereas this treatment enhanced the CD4$^+$ T cell responses to OVA, GAT, and TT only marginally (1.2-fold). These results demonstrate the presence of residual CD8$^+$ T cells in the CD4$^+$ T cell subpopulation and also identify a regulatory role for CD8$^+$ T cells in a CD4$^+$ T cell-mediated, insulin-specific response.

We next analyzed the insulin-specific proliferative responses of L.B.'s T cells obtained by the addition of increasing numbers of CD8$^+$ T cells to a constant number (10^5) of CD4$^+$ T cells (Fig. 2). Maximal suppression of the responses to HI and SBI was obtained upon the addition of 10^5 CD8$^+$ T cells (i.e., at a ratio of one CD8$^+$ T cell to one CD4$^+$ T cell). The addition of anti-CD8 to the latter T cell cultures reconstituted the insulin-specific proliferation to a level somewhat higher than that seen with anti-CD8-treated CD4$^+$ T cells. These data indicate that CD4$^+$ and CD8$^+$ T cells interact to regulate the net immune response to insulin in this patient. L.B.'s CD4$^+$ T cell responses to OVA (Fig. 2) and GAT (data not shown) were not affected by added CD8$^+$ T cells.

CD8$^+$ regulatory T cells in insulin-nonresistant type I diabetic patients. To determine whether CD8$^+$ T cells regulate insulin-specific responses in other type I diabetic individuals,

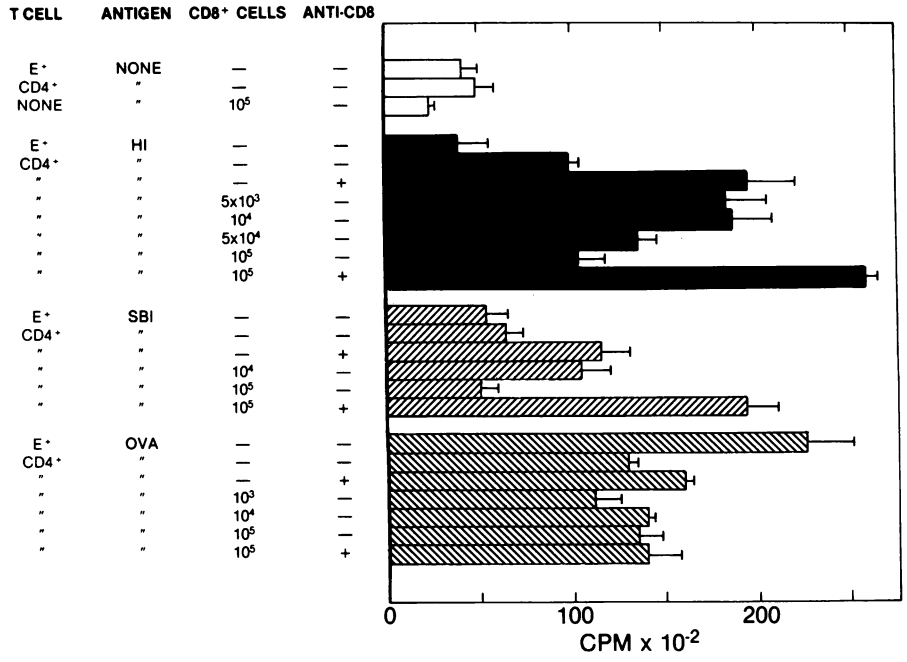

Figure 2. Regulation of an insulin-specific T cell proliferative response by patient L.B.[1] CD8$^+$ T cells. Primary in vitro T cell proliferative responses of either 10^5 E$^+$ or 10^5 CD4$^+$ (CD8-depleted) cells derived from an immunologically insulin-resistant patient (L.B.) to either HI (■), SBI (▨), or OVA (▨), used as a control antigen, were analyzed. These responses were compared with those obtained upon the addition of varying numbers of CD8$^+$ T cells to CD4$^+$ T cells. E$^-$ B cells (3–4 × 10^4) were added to each culture as APC. Antigens were used at the final concentrations of HI = 500 μg/ml, SBI = 500 μg/ml, and OVA = 1 mg/ml. Where indicated (+), an anti-CD8 MAb was added to culture at a final concentration of 0.5 μg/ml. Results are expressed as the mean of triplicate determinations, and error bars represent SD of the mean. Similar results were obtained in two experiments.

we performed similar CD4+ and CD8+ T cell mixing experiments with two other type I diabetic patients (A.K. and I.B.) who presented with high levels of anti-insulin antibodies but no immunologic resistance to insulin. Patient A.K. was a DR2/-, 26-yr-old female who presented with type I diabetes at the age of 16. She received treatment with Lente insulin (BI plus PI) before treatment with semisynthetic HI (0.73 U/kg per d). During 1988 she was found to have lipoatrophy. The concentration of her anti-insulin serum IgG antibodies was evaluated to be 3,440 μU/ml and her glycosylated Hb was < 8% (normal value, 4–8%). Patient I.B. was a DR2/-, 67-yr-old female who contacted type I diabetes 25 yr ago. She was treated with Lente insulin before receiving semisynthetic HI (0.44 U/kg per d). During 1988 she was diagnosed to have type I hypersensitivity to protamine sulfate, HI, BI, and PI, and these allergies were stabilized by treatment with Zaditen. The concentration of her anti-insulin serum IgG antibodies was determined to be 1,600 U/ml and her glycoslyated Hb was 8–12%. Patient G.S. was a 50-yr-old, nondiabetic obese female (HLA type not determined) who served as a control individual.

For these patients CD4+ and CD8+ T cell depletion was optimized using magnetic beads (see Methods) and checked by flow cytometry (> 95% pure CD4+ and CD8+ T cell populations were obtained). Our results demonstrate that both A.K. and I.B. displayed T cell reactivity to various forms of insulin, the highest response being obtained to BI (Fig. 3 *A*). This result was obtained in two (patient I.B.) or three (patient A.K.) separate experiments using either total T cells (Fig. 3, *A* and *D*) or purified CD4+ T cells (Fig. 3, *B*, *C*, and *E*). The level of reactivity to HI (100 μg/ml) was between two and five times the background level of proliferation.

When increasing numbers of CD8+ T cells were added to the CD4+ T cell/APC cell culture, we observed a progressive decrease in the CD4+ T cell response to BI (Fig. 3, *B* and *E*). CD8+ T cells did not proliferate in the presence of APC and antigen (data not shown). A maximum suppressive effect was observed when a ratio of 0.2 to 1 CD8+ T cell per CD4+ T cell was used. The CD8+ T cell–mediated suppression noted for patients A.K. and I.B. was not specific for BI since an inhibitory effect was also observed when GAT but not purified protein derivative (PPD) (Fig. 3 *C*) was used as antigens. This CD8+ T cell–mediated suppression to BI, GAT, and PPD was eliminated by the addition of anti-CD8 to cultures containing CD4+ T cells, CD8+ T cells, and APC (Fig. 3, *C* and *F*). This result is similar to that obtained for patient L.B. (Fig. 2), with the exception that L.B.'s CD8+ T cell–mediated suppressive effect was apparently specific for insulin. Note that the CD8+ T cell–mediated suppression of patient G.S. CD4+ T cell responses to GAT and PPD was less (Fig. 3 *F*) than that observed for patient A.K. (Fig. 3 *C*). Patient G.S., a healthy, nondiabetic individual, did not mount a significant in vitro CD4+ T cell response to BI.

Immunogenicity of SBI. Previously we reported that L.B.'s T cells recognized an immunodominant A-loop-associated epitope of HI (11). Sulfation of insulin may modify the conformation of this epitope and alter its immunogenicity (see Discussion). It was of interest, therefore, to analyze the in vitro responses to BI and SBI of BI A-chain-loop–reactive T cells derived from a group of individuals (both diabetic and nondia-

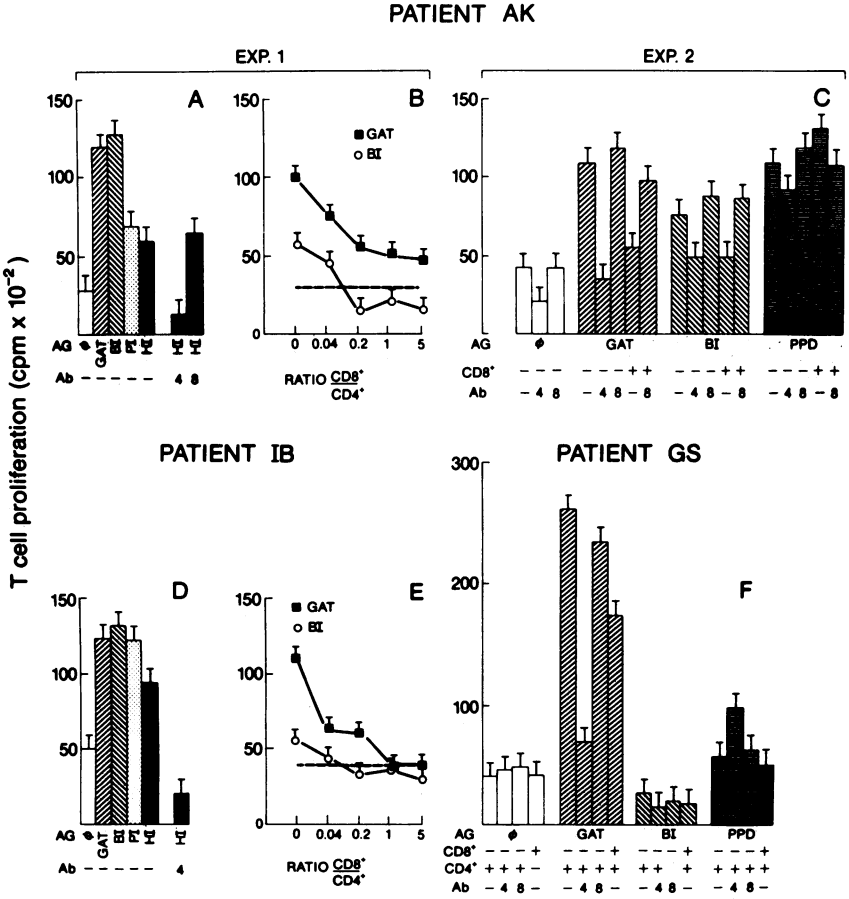

Figure 3. Regulation of insulin-specific T cell proliferative responses by CD8+ T cells from patients A.K., I.B., and G.S. Primary in vitro T cell proliferative responses of either 10^5 E+ (*A* and *D*) or 10^5 CD4+ (*B–F*) cells from two diabetic patients (A.K. and I.B.) and a nondiabetic individual (G.S.) to 300 (*A* and *D*) or 150 (*B–F*) μg/ml of BI (▨), PI (□), or HI (■), 1 mg/ml GAT (▨), or 10 μg/ml PPD (□) were analyzed. No antigen (○) control responses (□) were also assayed. CD4+ and CD8+ T cells were prepared by negative selection using magnetic beads. The varying ratios of CD8+/CD4+ T cells achieved in experiment 1 by the addition of increasing numbers ($0–5 \times 10^5$) of CD8+ T cells to 10^5 CD4+ T cells is shown. Ascites fluid containing anti-CD4 (13.B8.2) or anti-CD8 (10.D11.5) MAbs were titrated in each experiment; the indicated values represent the optimal conditions achieved using a 1:100 dilution of ascites fluid. The presence (+) or absence (−) of antigen (*AG*), anti-CD4 or anti-CD8 antibodies (*Ab*), and CD4+ or CD8+ T cells is indicated. Results are expressed as the mean of triplicate values, and error bars represent SD of the mean. The data shown in experiments 1 and 2 were reproducible in repeat experiments.

Table IV. SBI Is Nonimmunogenic for BI A-chain Loop-reactive T Cells

Patient	Haplotype (HLA-DR)	Type of diabetes	Proliferative response*				Immunodominant epitope‡
			BI	SBI	Control	Index	
			cpm × 10^{-2}				
G.E.	3/3	I	62±3	23±5	35±10	0	A-loop
M.S.	3/5	—	179±28	103±4	113±12	0	A-loop
L.B.	3/4	I	32±4	5±1	6±1	0	A-loop
K.S.	3/4	I	133±10	99±17	99±1	0	A-loop and B-chain
B.S.	4/4	I	166±16	66±9	56±1	0.1	A-loop and B-chain
R.S.	4/7	—	104±35	50±3	42±9	0.1	A-loop and B-chain
Re.S.	4/5	—	314±59	168±26	70±12	0.4	A-loop and B-chain
Mi.S.	4/4	—	342±2	226±1	113±10	0.5	A-loop and B-chain
D.E.	3/4	—	199±19	173±3	68±1	0.8	B-chain
B8P3§			172±2	4±1	2±1	0	A-loop

* T cell responses to SBI and BI were determined as in Table II in the antigen concentration range of 50–500 μg/ml. Results are expressed as the mean [^{3}H]TdR counts per minute of triplicate cultures ± SD of the mean. Similar results were obtained in three experiments. The values shown were those obtained at an antigen concentration of 250 μg/ml, which yielded a maximum level of response. The index of the relative T cell response of SBI to BI was calculated as follows: SBI response − control response/BI response − control response. Control responses were measured in the absence of added antigen. Statistically significant indices ($P \leq 0.01$) are underlined. ‡ Identification of the immunodominant BI epitope recognized by T cells from these individuals was determined by quantitating the responses of these PBL T cells to a panel of selected BI peptides (9). § B8P3 is a subclone of the previously described (29) mouse T cell hybridoma B8/C3X/B, which responds to BI and PI in association with I-A^d. The data presented here represent a measure of the antigen-induced release of IL-2 as assayed by the stimulation of [^{3}H]TdR incorporation by the mouse IL-2-dependent CTLL cytotoxic T cell line.

betic) who did not receive SBI treatment. T cells of individuals (including L.B.) that recognize an A-loop-associated determinant of HI respond very weakly or not at all to SBI (Table IV). A similar negative response to SBI was noted for a mouse T cell hybridoma, B8P3, which recognizes an A-loop epitope of BI (12). Interestingly, T cells from an individual (D.E.) that recognize B-chain-associated epitopes responded best to SBI. Intermediate responses to SBI were obtained with T cells from two individuals, Re.S. and Mi.S., who respond to both A-loop- and B-chain-associated epitopes. Thus, sulfation of BI reduces the T cell immunogenicity of an A-loop-associated epitope(s) to a much greater extent than the B-chain-associated epitopes.

Discussion

These results demonstrate that sulfation of BI reduces its immunogenicity for insulin A-loop-specific T cells in mouse and man. SBI was ineffective at stimulating the in vitro proliferation of T cells from 13 insulin-nonresistant type I diabetic patients. Our data also show that IIR in a type I diabetic patient may be abrogated by regulatory CD8$^+$ T cells elicited by her treatment with SBI. The emergence of these CD8$^+$ T cells coincided with an impairment of her CD4$^+$ T cell responsiveness to insulin and anti-insulin antibody production, both of which contributed to the elimination of her resistance to insulin therapy. It is interesting to extend these observations to an examination of other such insulin resistant diabetics, but such patients are rare. L.B. is the only type I diabetic in Canada whom we know is immunologically insulin resistant and who has been available to us for long-term immunological monitoring. Some immunologically insulin-resistant diabetics undergo spontaneous remission of this resistance without requiring SBI treatment (13). However, this was not the case for patient L.B.

The pattern of nonresponsiveness to insulin noted for patient L.B. is similar to that observed in H-2^b mice, which are genetic low responders to PI but possess both PI-specific helper T cells and dominant suppressor T cells that crossreact with mouse insulin (14, 15). It was proposed that these suppressor T cells recognize A-chain-loop–associated epitopes of pork and mouse insulin, and that their dominant effect results in the PI low responder phenotype of these mice (14, 16). Thus, in both humans and mice it is apparent that immunization with a nonimmunogenic form of insulin in a permissive MHC haplotype may activate dominant regulatory T cells that recognize autologous insulin. Suppressor T cells may inhibit the ability of helper T cells to stimulate B cells to produce antibodies (14), and suppressor T cell lines may block in vitro proliferation of antigen-specific helper T cells (17). L.B.'s CD8$^+$ T cells therefore seem to function as suppressor T cells, since the decrease in her anti-insulin antibody production paralleled the appearance of her CD8$^+$ T cells.

It was difficult to demonstrate insulin-specific suppressor activity of CD8$^+$ T cells obtained from 11 other insulin-nonresistant type I diabetics. Such patients may possess peripheral CD8$^+$ T cells that regulate their immune response to insulin, but the low frequency of such cells may preclude detection of their activity in vitro. In fact, the activity of such insulin-reactive regulatory CD8$^+$ T cells may vary with the age of a patient. It is of interest in this regard that the level of insulin autoantibody (IAA) production is a predictive marker for type I diabetes, but usually only before puberty (18). Younger children who are developing type I diabetes are more often IAA$^+$. Thus, older (postpuberty) individuals developing this disease generally have lower concentrations and titers of serum IAA, perhaps as a result of an increase in the activity of insulin-reactive CD8$^+$ T cells. After insulin treatment of type I diabetes patients is begun, these CD8$^+$ T cells may accumu-

late, become more effective with time, and thereby modulate the IAA response both in adult prediabetic individuals and in postpuberty type I diabetic patients possessing high titers of serum IAA. Thus, treatment of a type I diabetic individual with the nonimmunogenic SBI may simply accelerate the development of such $CD8^+$ T cells.

We did, however, detect the presence of $CD8^+$ regulatory T cells in two type I diabetic individuals, A.K. and I.B., who were maintained by treatment with HI, possessed high titers of anti-insulin serum antibodies, did not develop IIR, and displayed only a weak in vitro T cell response to insulin. The specificity of $CD8^+$ T cells from these patients was less restricted to insulin since these cells suppressed the response of $CD4^+$ T cells to both insulin and GAT, and to PPD to a lesser extent. Several mechanisms may account for this apparent lack of $CD8^+$ T cell antigen specificity. First, as has been observed in leprosy patients, both monocytes (antigen nonspecific) and $CD8^+$ T cells (antigen specific) can suppress immune responsiveness (19). Note that monocytes/macrophages were present in our cultures of selected $CD4^+$ and $CD8^+$ T cell subpopulations. Second, $CD8^+$ T cells may function as cytotoxic cells directed against either $CD4^+$ T cells (20) or antigen-specific B cells (21). For example, such cytotoxic $CD8^+$ T cells could recognize clonotypic receptors in the context of MHC class I molecules on $CD4^+$ T cells or insulin peptides in association with MHC class II molecules on B cells. The regulatory effect of $CD8^+$ T cells was more readily detected in patients such as A.K. and I.B. (both express DR2), and L.B. (DR3/4), each of whom possessed high titers of anti-insulin antibodies and consequently a greater number of insulin-specific B cells. We excluded the possibility that $CD8^+$ T cells simply absorbed all the IL-2 in culture required for the proliferation of $CD4^+$ T cells, since $CD4^+$ T cells grew well in cultures that contained $CD8^+$ T cells and to which graded numbers of IL-2-dependent CTLL cells were added. Third, the MHC haplotype of an individual may influence the responsiveness of her/his $CD8^+$ T cells. The differences in the specificities of the $CD8^+$ T cells observed between these patients could therefore be due to their expression of distinct MHC class II restricting elements (i.e., DR2 vs. DR 3/4). Fourth, our data are also similar to those reported recently for a $CD8^+$ T cell–mediated suppression of the expression of the Igh-1b allotype in mice. In this study the in vivo injection of an anti-CD8 antibody depleted the subset of $CD4^- 8^+$ peripheral T cells, increased the relative activity of helper T cells, and enhanced Igh-1b positive Ig production (22). In summary, these results are consistent with the idea that the net balance of activities of $CD4^+$ and $CD8^+$ T cells regulates the level of immune responsiveness to insulin in these patients.

Sulfation of insulin adds sulfate groups (average of six out of a possible eight per molecule) to side chains of threonine, serine, and tyrosine residues (23, 24). Certain of these modifications are situated in regions of the A- and B-chain that we have shown contribute to the formation of three immunodominant conformational epitopes of insulin recognized by human T cells (9). SBI is much less immunogenic both in vivo (23–25) and in vitro (this report) than either BI or PI. Addition of many negatively charged groups to insulin may alter its tertiary conformation, kinetics, and pathway of processing by an APC, or the capacity of a processed fragment(s) either to bind to HLA-D region encoded class II antigens or to be recognized with sufficient avidity by helper T cell antigen receptors. Based on our results obtained with the responses of A-chain-loop–reactive human and mouse T cells to SBI and BI (Table IV), we favor the possibility that a change in the conformation of the A-loop epitope of SBI is largely responsible for its reduced immunogenicity. This proposed change in conformation is also supported by the demonstration that the insertion of negatively charged residues into human insulin, by site-specific mutagenesis at some of the same residues (e.g., B9, B26, B27) that may be modified in SBI, converts insulin from a multimeric to a monomeric form that is absorbed two to three times faster after subcutaneous injection in vivo (26).

SBI is currently manufactured by treatment of BI with H_2SO_4 for 20 min at room temperature (23, 27). When SBI is chromatographed by HPLC on a reverse-phase C18 column using various buffer systems, several poorly resolved peaks are observed (Naquet, P., unpublished observations). It has not been possible to identify which of these peaks is the active moiety or to establish whether all or only some of the BI residues listed above need to be sulfated to abolish the immunogenicity of BI in humans. Since we and others have shown that recombinant HI can on occasion provoke T cell and IgG antibody activity to insulin in type I diabetics, it would be advantageous to introduce structural modifications in this product to render it less immunogenic. In conclusion, the data presented here point the way for future attempts to modify HI either chemically or by site-specific mutagenesis (26) to derive a hormonally active and nonimmunogenic form of insulin to improve the maintenance of type I diabetic patients.

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