

# T-Cell Lines Reactive to an Immunodominant Epitope of the Tyrosine Phosphatase-Like Autoantigen IA-2 in Type 1 Diabetes

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Type 1 diabetes is the result of destruction of the insulin-secreting  $\beta$ -cells of the pancreas by a process in which T-cells play a central role. A tyrosine phosphatase-like protein, IA-2, is a major target for autoantibodies and T-cells in the disease. In this study, we have further characterized the T-cell response to IA-2 by the generation and characterization of T-cell lines. T-cell lines responsive to IA-2 antigen were generated from 17 of 32 patients and 3 of 10 control subjects. Antigen specificity was confirmed in lines from six diabetic patients and one control individual by demonstration of responses to synthetic IA-2 peptides and epitope mapping. Five lines from diabetic patients responded to one of two peptides representing amino acids 831–850 and 841–860 of IA-2. The overlapping portion may therefore represent an immunodominant region of the molecule. The sixth patient-derived line responded to a peptide representing amino acids 751–770 of IA-2 presented by the DR 4 (DRB1\*0401) allele that confers susceptibility to type 1 diabetes. Primary T-cell responses to peptides of the immunodominant region were detected in 9 of 19 (47%) type 1 diabetic patients and 16 of 22 (73%) nondiabetic siblings, consistent with this region having immunostimulatory properties. The study reports for the first time T-cell lines reactive to IA-2 from diabetic patients and defines an immunodominant region of the molecule. *Diabetes* 49:356–366, 2000

**T**ype 1 diabetes is a genetically associated autoimmune disease in which the insulin-producing pancreatic  $\beta$ -cells are destroyed by a process involving autoreactive T-cells (1). The majority of patients develop humoral immune responses to defined islet cell autoantigens including insulin (2), the 65-kDa isoform of

GAD65 (3), ICA69 (4), and two tyrosine phosphatase-like proteins designated IA-2 (or ICA512) and IA-2 $\beta$  (or phogrin), of which IA-2 appears to be dominant (5–9). IA-2 antibodies are detected in 60–70% of type 1 diabetic patients and are associated with rapid progression to diabetes in relatives of patients (10,11). IA-2 antibodies are more prevalent in patients with younger age of disease onset and in patients with diabetes susceptibility HLA-DR4 alleles (12). Epitopes for IA-2 antibodies are found exclusively within the cytoplasmic region of the molecule and predominantly within the tyrosine phosphatase-like domain, which shows a high degree of homology in IA-2 $\beta$  (9,13,14).

While antibody responses to islet cell autoantigens have been extensively characterized, relatively little is known about T-cell reactivity to islet components in the disease. This situation is, in part, a consequence of difficulties in gaining access to those T-cells infiltrating the pancreatic islets in the disease. A number of studies have examined the possibility that peripheral blood lymphocytes can be a source of disease-related T-cells and proliferative responses of circulating T-cells have been reported to insulinoma membrane preparations (15) and to purified insulin (16), GAD (17,18), ICA69 (19), and IA-2/ICA512 (20,21). More type 1 diabetic patients than healthy control subjects respond to these preparations, thus providing evidence of a disease-related T-cell response to the autoantigens studied. The aim of this study was to further characterize the T-cell response to IA-2 by stimulating peripheral blood lymphocytes from diabetic and control individuals with recombinant protein representing the cytoplasmic domain of IA-2, followed by expansion of activated T-cells with interleukin-2 to generate T-cell lines. Synthetic peptides were used to confirm antigen specificity and to map regions recognized. Peptides recognized by T-cell lines were then used to further analyze T-cell reactivity in diabetic and nondiabetic populations.

## RESEARCH DESIGN AND METHODS

**Subjects.** Blood was obtained with informed consent by venepuncture from 19 children (mean age  $7.8 \pm 4.2$  years, range 1–16) with newly diagnosed type 1 diabetes (duration <4 weeks) from the southwest of the Netherlands and from 23 patients attending the Diabetes Clinic of King's College Hospital, London, U.K. (mean age  $22.5 \pm 1.9$  years, range 8–39, disease duration <6 months). A total of 21 first-degree relatives of type 1 diabetic patients from the Netherlands (mean age  $7.3 \pm 3.6$  years, range 1–14) and 10 healthy volunteers with no family history of type 1 diabetes (mean age  $25.4 \pm 2.8$  years, range 10–39) served as nondiabetic control subjects. All nondiabetic control subjects were negative for islet cell antibodies (ICA) and antibodies to GAD65 and IA-2. Subjects were typed for HLA class II alleles by hybridization with sequence-specific oligonucleotides after DNA amplification with DR- and DQ-specific primers (22,23). Serum was used for analysis of antibodies

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cpm, counts per minute; ICA, islet cell antibodies; IL-2, interleukin-2; MHC, major histocompatibility complex; M<sub>r</sub>, relative molecular weight; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SI, stimulation index.

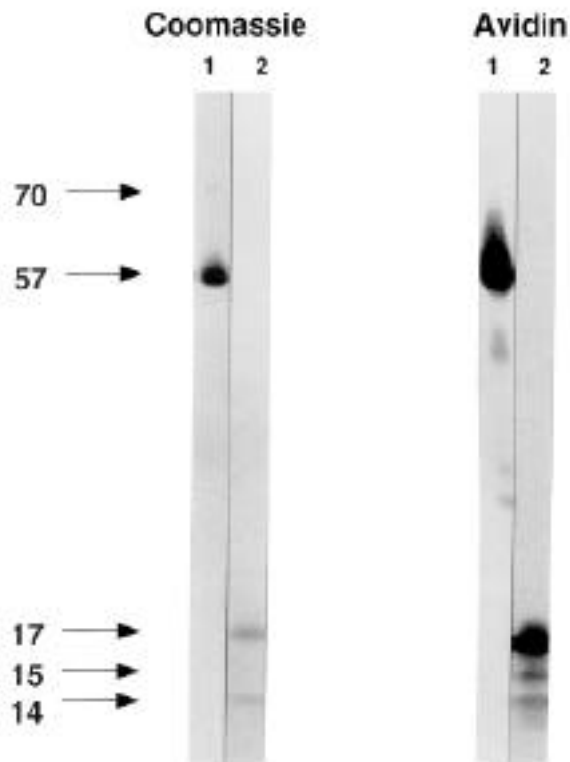


FIG. 1. Purity of antigen preparations for T-cell studies. IA-2ic (lane 1 in figure) or control antigen (lane 2) were purified from *E. coli* extracts by affinity chromatography. The purity of the preparations was determined by Coomassie staining (left panel), Western blotting or with alkaline phosphatase-conjugated avidin using 5-bromo, 4-chloro, 3-indolyl phosphate/nitroblue tetrazolium substrate (right panel). The arrows mark the  $M_r$  ( $\times 10^{-3}$ ) of the major components of the preparations.

to IA-2 using a radioligand binding assay (11). Antibody levels were expressed as an antibody index relative to a standard positive serum included in each assay, and sera with an antibody index  $>0.1$  were considered positive.

Antigens. cDNA for the cytoplasmic domain of IA-2 (IA-2ic, representing amino acids 605–979) was cloned into the Pinpoint (Promega, Southampton, U.K.) expression vector for expression in *Escherichia coli* as a fusion protein with a biotinylated purification tag at the amino terminus (6). A Pinpoint vector encoding the biotinylated purification tag sequence alone was used for the expression of a control protein for T-cell studies. Proteins were expressed in *E. coli* and extracted in buffer containing 10 mmol/l benzimidazole and 1 mmol/l PMSF as protease inhibitors. Extraction was performed using 1 mg/ml of lysozyme for 20 min, followed by lysis for 5 min in the presence of 0.1% Triton X-100. The viscosity of the resulting extract was reduced by incubating with 200 U of DNase I for 10 min. Cellular debris was removed by centrifugation at 8,000g for 10 min at 4°C.

IA-2ic or control antigen was purified from the bacterial extract on an avidin affinity column and eluted with 5 mmol/l biotin. Proteins were dialyzed extensively against 1 mmol/l sodium phosphate, pH 7.4, lyophilized, and stored at  $-70^\circ\text{C}$ . The purity of eluted proteins was determined by SDS-PAGE analysis and Coomassie staining and by Western blotting with alkaline phosphatase-conjugated avidin, or a monoclonal mouse antibody to IA-2 (antibody 76F; a kind gift of Dr. E. Bonifacio, Milan, Italy) combined with a secondary antibody to mouse IgG (Sigma, Poole, Dorset, U.K.), and detection using 5-bromo, 4-chloro, 3-indolyl phosphate and nitroblue tetrazolium substrate.

For determination of epitope specificity, a series of 37 20-mer peptides and one COOH-terminal 19-mer peptide (p1–p38), each overlapping by 10 residues and spanning the entire cytoplasmic domain of the IA-2 molecule (amino acids 591–979), was synthesized as described (24). Fine mapping of epitopes was performed with appropriate 16-mer peptides synthesized in a similar manner.

The protein content of each protein preparation was determined using a protein assay kit (Pierce, Chester, U.K.), and all preparations were filter-sterilized using low-protein binding filters (Millipore, Watford, Herts, U.K.) before use in T-cell stimulation experiments. The endotoxin content in the IA-2ic preparation was determined to be  $<3$  endotoxin units per 5  $\mu\text{g}$ .

Generation of T-cell lines. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation and incubated at a cell density of  $10^6$  cells per milliliter in tissue culture plates in RPMI-1640 culture medium containing 10% autologous or pooled AB<sup>+</sup> human serum and IA-2ic at a concentration of 5–10  $\mu\text{g}/\text{ml}$ . Cells were incubated at 37°C with 5% CO<sub>2</sub> for 4–6 days and then expanded in tissue culture medium containing 20 U/ml interleukin-2 (IL-2). After culturing for a further 5–7 days, the cells were restimulated with either IA-2ic or phytohemagglutinin (PHA) in the presence of irradiated PBMC as feeder cells. After 3–4 days stimulation, the cells were further expanded with IL-2 and then tested for antigen specificity in a lymphocyte proliferation assay. Cells were cloned at 0.3 cells per well as previously described (25).

Antigen specificity of T-cell lines and clones was tested by incubating  $10^4$  T-cells with  $5 \times 10^4$  irradiated PBMCs in 150  $\mu\text{l}$  of culture medium in 96-well tissue culture plates. Cells were incubated in triplicate with medium alone or with IA-2ic, IA-2ic peptides, control purification tag protein, PHA, or IL-2 at concentrations indicated in the text or figure legends. In blocking studies to establish HLA restriction, antibodies to HLA-DR (B8.11.2, IgG2b), HLA-DQ (SPV-L3, IgG2a), and HLA-DP (B7.21, IgG2a) were added at 10  $\mu\text{g}/\text{ml}$  as described (26). After 3 days incubation, 0.5  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine (Amersham International, Amersham, U.K.) was added in 50  $\mu\text{l}$  medium and incubation continued for 16 h. Cultures were harvested onto glass fiber filters and [<sup>3</sup>H]thymidine incorporation measured by  $\beta$ -counting. The results of lymphocyte proliferation are expressed as stimulation indices (SI), defined as counts per minute (cpm) in presence of antigen divided by cpm in medium alone.

Flow cytometric analysis was performed using fluorescein isothiocyanate-labeled antibodies (Becton Dickinson, San Jose CA) to CD4 (T-helper cell) and CD69 (activation marker) and PE-labeled antibodies (Becton Dickinson) to CD8 (T-cytotoxic) and CD56 (natural killer cell) as described previously (27).

Lymphocyte stimulation assays. PBMCs were tested in a primary lymphocyte stimulation assay as previously described (28) using culture medium alone or supplemented with IA-2ic, IA-2, GAD, or proinsulin peptides, control antigen, PHA, IL-2, or tetanus toxoid at concentrations indicated in the text. Cells were incubated in triplicate for 5 days before addition of 0.5  $\mu\text{Ci}$  of [<sup>3</sup>H]-methyl-thymidine, then incubated for a further 16 h and harvested onto a glass fiber filter. Results were expressed as SI and proliferative responses were considered positive for SI  $>3$ . Statistical analysis. The magnitude of T-cell proliferative responses between groups was compared using the Mann Whitney U test and differences in frequencies of positive T-cell responses using Fisher's exact test. Relationships between T-cell proliferation and different antigens were determined by Spearman rank correlation. Statistical analysis was performed using GraphPad InStat or Prism computer software (GraphPad Software, San Diego, CA).

## RESULTS

Purification of antigens. Protein representing the cytoplasmic domain of IA-2 (IA-2ic), the region recognized by antibodies in type 1 diabetes, was purified by avidin affinity chromatography as a fusion protein with a biotinylated Pinpoint purification tag from bacterial extracts. The Pinpoint purification tag itself was also expressed and purified as a control protein for the T-cell studies. The purity of the extracts was analyzed by SDS-PAGE, Coomassie brilliant blue staining, and Western blotting. Coomassie staining of gels of the IA-2ic preparation showed the IA-2ic migrating as a major band of relative molecular weight ( $M_r$ ) 57,000, with a single visible contaminant at  $M_r$  70,000 (Fig. 1). Densitometric scanning of gels indicated that the  $M_r$  57,000 band represented  $>90\%$  of the protein in the sample. Western blotting with alkaline phosphatase-conjugated avidin, or with a monoclonal antibody to IA-2, revealed minimal proteolytic degradation of the IA-2 preparations, with only three weak bands between  $M_r$  30,000 and 50,000 visible (Fig. 1). The  $M_r$  70,000 contaminating protein was not detected by either the avidin or the IA-2 antibody probes. The control purification tag preparation migrated as two bands of 14,000 and 17,000  $M_r$ , both of which bound avidin, and there was only one visible contaminant of  $M_r$  15,000 visible by avidin blotting.

Generation of T-cell lines. Peripheral blood lymphocytes from 32 type 1 diabetic patients (9 from the Netherlands and 23 from the U.K.) and 10 healthy control subjects (all

TABLE 1  
Generation of IA-2-responsive T-cell lines

	Age	Sex	HLA-DR	HLA-DQ	IA-2 antibodies	IA-2 response (SI)	Dominant peptide response
Diabetic patient							
TL	15	M	1/4	NT	Pos	11	p17
RG	14	M	4/5	7/8	Pos	124	p25 + p26
YB	12	M	3/4	2/8	Pos	67	p25 + p26
BM	5	M	1/3	1/5	Neg	68	p25 + p26
MB	12	F	9/10	2/5	Pos	5	p25
RM	24	F	3/9	NT	Pos	5	p26
AJ	14	F	4/11	7/8	Pos	28	None
AW	30	M	NT	NT	Pos	25	None
JH	16	M	3/9	2/9	NT	47	None
FB	8	M	3/3	2	NT	18	None
JJ	38	M	3	NT	Pos	11	None
SC	31	M	4/8	8	Pos	125	NT
SH	26	F	3/4	2/8	Pos	30	NT
TC	31	M	1/4	5/8	Neg	224	NT
NP	29	M	1/6	5/6	Pos	62	NT
CM	8	F	3/4	2/8	Neg	30	NT
TY	21	M	3/4	2/8	Pos	12	NT
EB	13	M	4/13	3/6	Neg	<3	—
KG	8	M	1/4	2/5	Pos	<3	—
RB	1	M	4/4	8	Pos	<3	—
AZ	9	F	3/4	2/8	NT	<3	—
AC	8	M	4/6	5/8	Pos	<3	—
SG	20	M	4	8	Pos	<3	—
SS	23	M	1/4	5/8	Neg	<3	—
KB	11	F	4/13	5/8	Pos	<3	—
PO	28	M	9/15	2/6	Neg	<3	—
MI	16	F	12/13	6/7	Pos	<3	—
AA	16	F	3/10	2	Pos	<3	—
PW	39	M	4/11	NT	Neg	<3	—
JD	28	M	1/3	2/5	Neg	<3	—
ML	29	M	4	NT	Pos	<3	—
PQ	20	M	3	2	Neg	<3	—
Control subject							
NS	25	M	4	7	Neg	311	Pool p21–25
CA	17	F	4/16	5/6	Neg	55	None
EM	20	M	1/11	5/7	Neg	100	None
CH	24	F	1/7	5/9	Neg	<3	—
JT	28	F	15	1	Neg	<3	—
DC	10	M	15/16	5/6	Neg	<3	—
JM	37	M	1/15	5/6	Neg	<3	—
LH	24	F	1	NT	Neg	<3	—
ES	30	F	10/15	5/6	Neg	<3	—
MC	39	M	15/16	5/6	Neg	<3	—

T-cell lines were generated from diabetic and control subjects (identified by two-letter code) by cycles of stimulation of peripheral blood lymphocytes with IA-2ic and IL-2 and analyzed for proliferative response to IA-2ic at concentrations of 0.01–10 µg/ml. The maximum response of each line is expressed as SI. T-cell lines with positive responses were tested for reactivity with synthetic IA-2 peptides, and peptides showing dominant reactivity with lines are listed. NT, not tested.

from the U.K.) were used to generate T-cell lines by stimulation with IA-2ic and IL-2. After two rounds of stimulation, T-cell lines were analyzed for reactivity with IA-2ic in a proliferation assay. T-cell lines showing strong proliferative responses to IA-2ic (SI = 5) were obtained from 17 of the 32 type 1 diabetic patients and from 3 of the 10 healthy controls (Table 1). The ability to generate lines to the preparation was not associated with the presence or absence of serum antibodies to IA-2, nor with the expression of specific HLA-DR or -DQ alleles in the patient.

Specificity of IA-2ic-reactive T-cell lines. To investigate the specificity of the T-cell response to IA-2, T-cell lines reac-

tive to the IA-2ic preparation were analyzed for their ability to respond to a series of overlapping 20-mer synthetic IA-2 peptides. The peptides were tested initially as pools of 2–5 synthetic peptides, each at a concentration of 2 µg/ml. Of 11 IA-2ic-responsive lines from diabetic patients tested, 6 were found to respond to IA-2 peptides (Table 1 and Fig. 2). One of these (line TL) was found to respond to a pool of five peptides (p16–p20) representing amino acids 741–800 of the IA-2 molecule (Fig. 2A). Analysis of reactivity to individual peptides within this pool indicated a predominant reactivity to peptide p17, representing amino acids 751–770. The remaining five lines (RG, BM, YB, MB, and RM) all responded to pools con-

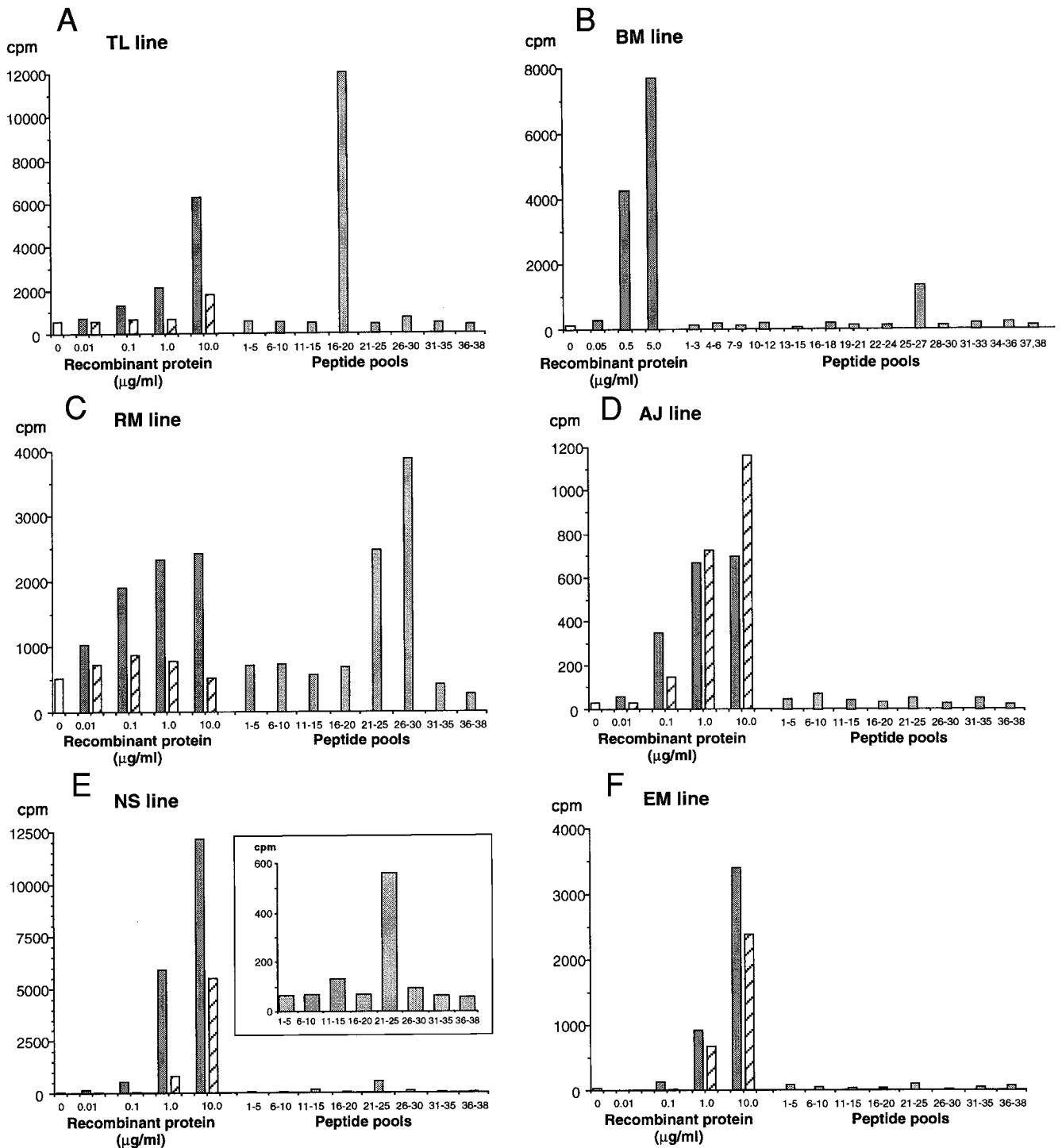


FIG. 2. Antigen responses of T-cell lines. T-cell lines were generated by stimulation of peripheral blood lymphocytes with IA-2 and IL-2, expanded and analyzed for proliferative responses in medium alone ( $\square$ ) or in the presence IA-2ic ( $\blacksquare$ ) or control recombinant protein ( $\boxtimes$ ) at concentrations between 0.01 to 10  $\mu\text{g/ml}$ . Lines were also tested for proliferative responses to pools of 2–5 synthetic IA-2 peptides, each at 2  $\mu\text{g/ml}$ . The figure shows the results of four representative lines from diabetic patients (A–D) and two lines from healthy control subjects (E and F). The BM line was not tested against control protein. The peptide response of the NS line is presented with an expanded scale in the inset figure (E). Results are plotted as cpm of [ $^3\text{H}$ ]thymidine incorporated for each condition and are means of triplicate observations.

taining peptides in the region of amino acids 791–900 (Fig. 2B and C). When tested against individual peptides within relevant pools, these lines responded to at least one of two overlapping peptides, p25 (amino acids 831–850) and p26 (841–860) (Table 1).

Three T-cell lines with strong proliferative responses to p25 and p26 (lines RG, BM, and YB) were tested against the entire panel of 38 peptides spanning the intracellular domain of IA-2. Proliferative responses to the individual peptides confirmed the dominant reactivity against the p25 and p26

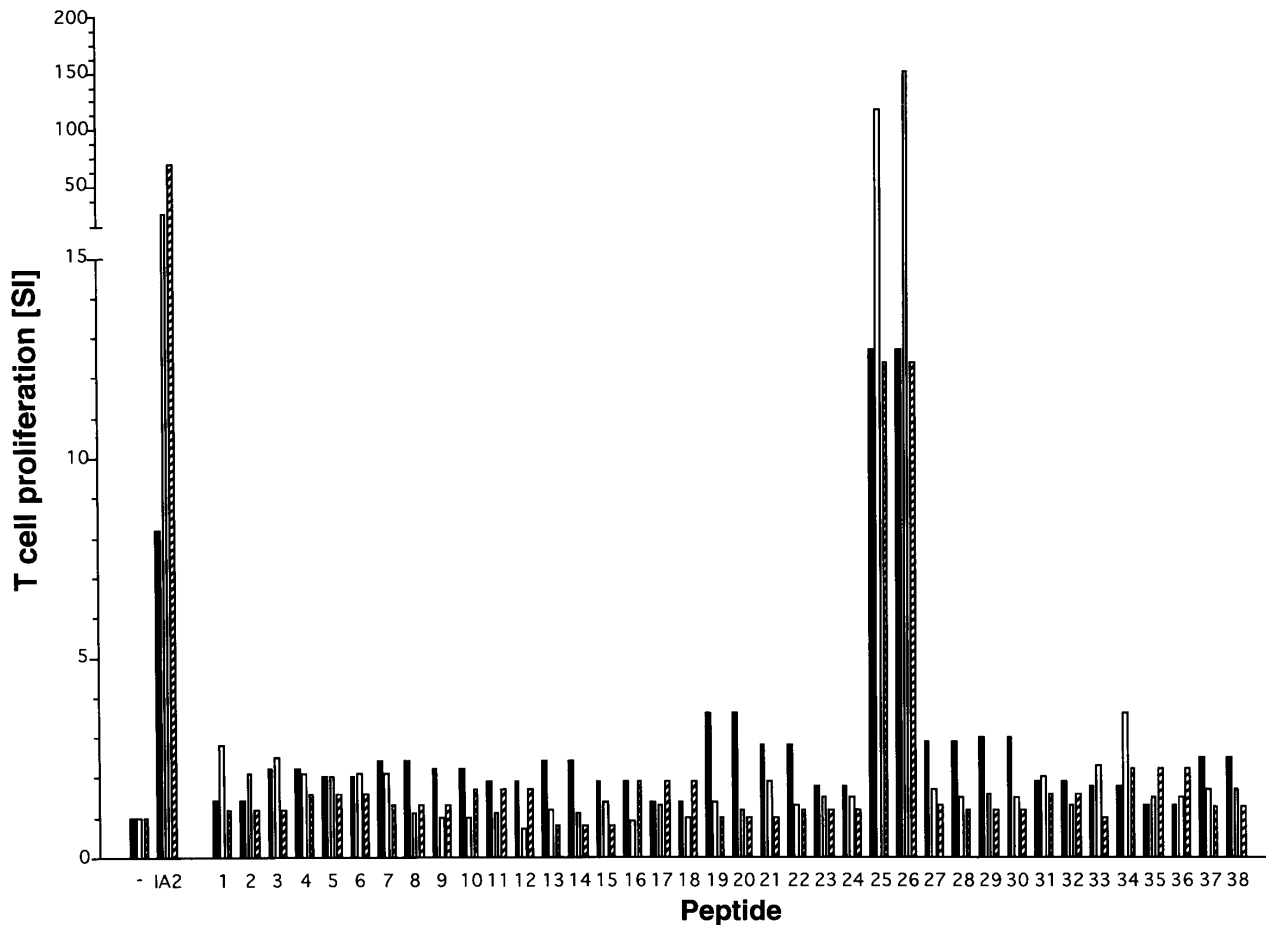


FIG. 3. Epitope mapping of T-cell lines. T-cell lines were analyzed for reactivity to individual synthetic peptides spanning the cytoplasmic domain of IA-2. Reactivity of line BM (■), YB (□), and RG (▨) to each peptide is plotted as SI.

peptides, since only weak responses were detected to other peptides in two of the three lines (Fig. 3). The fine specificity was mapped using 16-mer peptides with 10 amino acid overlaps spanning the region covered by p25 and p26. The minimal epitope was contained within the 16-mer peptide representing amino acids 841–856. In addition, T-cell line RG responded weakly (SI of ~3) to peptides p19 and p20 (771–790 and 781–800), and line BM showed weak responses to peptide p34 (921–940). These three IA-2-specific T-cell lines were cloned in the presence of PHA and IL-2 to obtain one (line RG), two (BM), and six (YB) T-cell clones, respectively, for each line. All clones recognized specifically the peptides p25 and p26, in common with the lines from which they were derived. Two of the T-cell clones of patients RG and BM were tested with IA-2ic protein, yielding proliferative responses of SI 14.0 and 29.7, confirming the notion that the epitope is indeed one that is derived by natural processing. Flow cytometric analysis of the T-cell clones indicated that these expressed CD4, but not CD8 or CD56, consistent with an activated T-helper phenotype.

Of the 11 T-cell lines from diabetic patients that responded to IA-2 ic preparation, 5 failed to respond to any of the synthetic peptide pools. To test for potential reactivity against non-IA-2 components of the purified recombinant protein preparation, T-cell lines were also tested for their reactivity to a control protein representing the biotinylated Pinpoint

purification tag, expressed and purified in a similar manner to the IA-2ic antigen. IA-2 peptide-responsive T-cell lines from diabetic patients showed only weak responses to this control antigen (Fig. 2A and C). In contrast, IA-2ic reactive T-cell lines that lacked reactivity to pools of IA-2 peptides did show proliferative responses to the control protein (Fig. 2D), suggesting that these lines were stimulated predominantly by the purification tag sequence or to bacterial contaminants in the recombinant protein preparations.

Three IA-2-responsive T-cell lines were obtained from nondiabetic control subjects. All three lines also responded strongly to the control preparation (Fig. 2E and F); only one (NS) showed a relatively weak response (SI of 14) compared with recombinant IA-2ic protein (SI 311) to one of the peptide pools containing peptides p21–p25, representing amino acids 791–850 (Fig. 2E inset). On further expansion of the T-cell line to obtain sufficient cells for testing reactivity to individual peptides within this pool, the peptide specificity of this line was lost.

HLA restriction of T-cell lines. IA-2 peptide-specific T-cell lines from four diabetic patients (TL, RG, BM, and YB) were used in blocking studies with monoclonal antibodies to determine which major histocompatibility complex (MHC) class II molecules present antigen to the line. Proliferation of the TL line in response to IA-2ic was effectively blocked by antibodies to HLA-DR, whereas antibodies to HLA-DQ and

TABLE 2

Proliferative responses of T-cell lines to IA-2ic in the absence or presence of monoclonal antibodies to HLA-DR, -DP, or -DQ

Line	Background (cpm)	Response to IA-2ic (cpm)	IA-2ic + HLA-DR	IA-2ic + HLA-DP	IA-2ic + HLA DQ
TL	382 ± 37	8,281 ± 892	531 ± 30	6,355 ± 369	5,183 ± 310
RG	165 ± 43	20,426 ± 2,227	19,146 ± 1,277	74 ± 15	20,969 ± 188
YB	152 ± 20	10,139 ± 113	8,429 ± 279	404 ± 155	11,459 ± 375
BM	72 ± 17	4,936 ± 1,404	4,220 ± 729	205 ± 60	4,809 ± 609

Data are mean cpm ± SE of triplicate observations.

-DP had minimal effect (Table 2). In contrast, the proliferative responses of three lines reactive to the p25 and p26 peptides (RG, BM, and YB) were all blocked by antibodies to HLA-DP, but not by antibodies to HLA-DR or HLA-DQ (Table 2).

To further characterize HLA restriction of the T-cell lines, proliferation assays were performed with a panel of antigen-presenting cells derived from donors partially matched for the HLA alleles of the patients. The HLA-DR-restricted T-cell line TL was obtained from a patient expressing DRB1\*0103 and DRB1\*0401 alleles. T-cell responses to IA-2ic were found to be maintained in the presence of PBMCs from donors expressing the DRB1\*0401 allele, but not in the presence of DRB1\*0103-positive donors lacking the DRB1\*0401 allele (Table 3), suggesting that the line is DRB1\*0401-restricted. The three patients from whom the HLA-DP-restricted p25/p26 reactive T-cell lines were derived all expressed the HLA-DPB1\*0401 allele. Responses of the T-cell lines to IA-2ic or the

synthetic peptides p25 and p26 were all maintained in the presence of antigen-presenting cells positive for HLA-DPB1\*0401 (Table 3), consistent with this allele being the restricting element. PBMCs of one HLA-DP4-positive donor (donor 10) were unable to present the epitope to T-cells of patient YB, although they did present via the same HLA-DP4 restriction element to T-cells of patient RG. Possible explanations for this discrepancy include micro-heterogeneity in DPB1\*0401 alleles, differences in processing by antigen presenting cells of different donors, or competitive effects of other MHC molecules.

T-cell responses to IA-2 peptides and control antigens. The finding that five of the six T-cell lines from diabetic patients that responded to IA-2 peptides reacted predominantly to peptides p25 or p26 suggested that these might be representative of an immunodominant region of the IA-2 molecule. Primary proliferation assays using PBMCs from dia-

TABLE 3

Proliferation of T-cell lines in response to IA-2ic (or p25 and p26 peptides) in the presence of PBMCs partly matched with the donor for MHC class II alleles

	HLA			Proliferation to IA-2 (cpm)
	DR	DQ	DP B1	
TL	<u>0103, 0401</u>			
Donor 1	0103, 1101	0301, 0501	0901, 1401	672 ± 56
Donor 2	0103, 1501	0501, 0602	0301, 0401	1,121 ± 127
Donor 3	<u>0401, 0403</u>	0302, 0303	NT	22,363 ± 1,186
Donor 4	0101, <u>0401</u>	0501, 0302	0401, 1901	25,579 ± 827
Donor 5	<u>0401</u>	0301, 0302	NT	37,335 ± 2,149
RG	4,12	7,8	0401	
Donor 6	11,12	7	<u>0401, 0402</u>	19,900 ± 1,433
Donor 7	4,7	7,9	0201, <u>0401</u>	5,300 ± 1,714
Donor 8	3,4	2,8	0201, <u>0401</u>	640 ± 432
Donor 9	12,13	6,7	0101, <u>0401</u>	19,080 ± 1,139
Donor 10	4,15	6,8	0201, <u>0401</u>	13,585 ± 5,284*
Donor 11	3,4	2,7	0301, 1501	977 ± 292
BM	1,17	2,5	<u>0401</u> , 1301	
Donor 12	1,7	2,5	<u>0401</u> , 1101	30,335 ± 2,915
Donor 13	3,4	2,8	0101, 0402	102 ± 67
Donor 8	3,4	2,8	0201, <u>0401</u>	25,172 ± 4,865
YB	3,4	2,8	<u>0401</u> , 0402	
Donor 14	3	2	0201, 0301	144 ± 77
Donor 15	3	2	<u>0401</u>	7,676 ± 760
Donor 10	4,15	6,8	0201, <u>0401</u>	82 ± 5
Donor 16	1,4	5,8	0301, 0402	316 ± 93
Donor 17	13,9	6,9	0201, <u>0401</u>	3,345 ± 1,836

Data are mean cpm ± SE of triplicate observations. \*T-cell line tested with p25/p26 peptides. The probable presenting allele for each line is underlined.

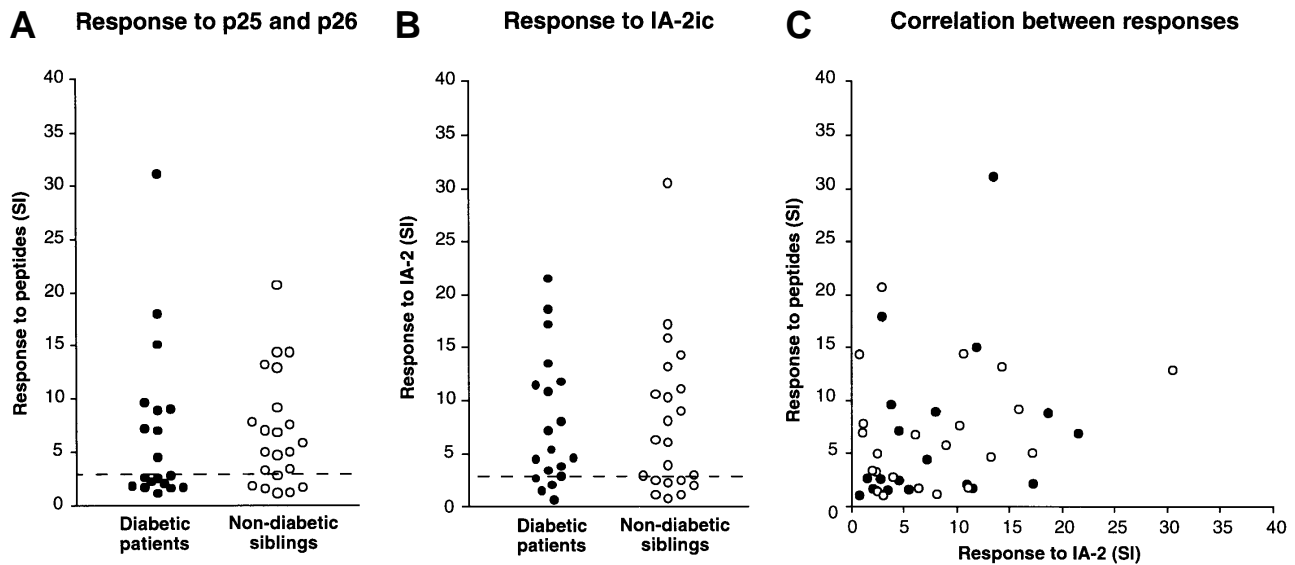


FIG. 4. Primary T-cell responses to peptides p25 and p26 and IA-2ic. Primary proliferative responses to a pool of peptides p25 and p26 (A), IA-2ic (B), and the relationship between the responses (C) are shown for recent-onset type 1 diabetic patients (●) and nondiabetic siblings (○).

betic patients and nondiabetic control individuals were performed to further investigate the T-cell response to this region of IA-2. Nondiabetic siblings of type 1 diabetic patients, sharing MHC class II alleles with the diabetic proband, were used as a control group for these experiments. T-cell responses to the two pooled synthetic peptides p25 and p26 were compared with those to the recombinant IA-2ic molecule. Relatively strong proliferative responses to peptides p25 and p26 were detected in both diabetic patients and nondiabetic siblings. Positive T-cell responses ( $SI > 3$ ) to the two pooled peptides were detected in 9 of 19 (47%) of the type 1 diabetic patients and in 16 of 22 (73%) of the nondiabetic siblings (Fig. 4A). The magnitude of responses to the synthetic peptides was similar to those detected to the recombinant IA-2ic protein (Fig. 4B). Similar to the results with the peptides, both type 1 diabetic patients (14 of 19; 79%) and nondiabetic siblings (13 of 22; 59%) recognized the recombinant protein (Fig. 4B). However, no significant association was observed between T-cell reactivity to the recombinant IA-2 preparation and to the two peptides (Fig. 4C). Interestingly, a significantly higher proportion of the siblings (7 of 22; 32%) than of type 1 diabetic patients (1 of 19; 5%) responded to the IA-2 peptides in the absence of a response to the IA-2 protein (Fig. 4C) ( $P < 0.05$ ), potentially indicative of reactivity to cryptic epitopes in the nondiabetic subjects.

Protein and peptide antigens were also included in T-cell proliferation studies as control stimuli. T-cell responses to tetanus toxoid were not different between patients and control subjects (mean stimulation indices  $22.3 \pm 13.5$  and  $34 \pm 31.7$ , respectively). Diabetic patients were also analyzed for proliferative responses to peptides representing regions of other autoantigens implicated in diabetes pathogenesis, including proinsulin (pool of 3 peptides spanning insulin B-chain residue 20 to C-peptide residue 14) (29), GAD65 PEVKEK region (pool of 5 peptides spanning amino acids 247–280) (30,31), and GAD65 peptide 339–352 (32,33). The strength of the T-cell response to IA-2 peptides was higher (mean  $SI \pm SE$ :  $8.2 \pm 6.3$ ) than that to the proinsulin ( $3.4 \pm 0.88$ ;

$P < 0.05$ ), GAD65 PEVKEK ( $1.5 \pm 0.7$ ), or GAD 339–352 ( $3.2 \pm 1.7$ ) peptides, consistent with the 831–860 region of IA-2 having particularly strong immunostimulatory properties. Proliferation of PBMCs in presence of medium alone (autologous response in the absence of exogenous stimulus) was not different between patients and control subjects.

Sera from all type 1 diabetic patients and siblings in this series were tested for antibodies to IA-2ic using a radioligand binding assay. Of the 19 type 1 diabetic patients in this series, 10 (53%) were positive for IA-2 antibodies, whereas all nondiabetic siblings were negative. No correlation was detected between levels of antibodies to IA-2ic and the primary T-cell response to IA-2 peptides (Fig. 5A) or to IA-2ic (Fig. 5B) in the diabetic patients.

#### DISCUSSION

A number of groups have demonstrated diabetes-associated responses of peripheral blood lymphocytes to islet cell antigen preparations, ranging from crude extracts of islets and insulin-secreting cell lines to highly purified recombinant islet proteins (15–21). The presence of circulating T-cells to islet cell autoantigens in type 1 diabetes is most convincingly demonstrated by the recent generation of T-cell lines to purified GAD that respond to synthetic peptides representing different regions of the GAD molecule (34,35). We now report the generation of T-cell lines to naturally processed epitopes on the tyrosine phosphatase-like protein IA-2 by stimulation of peripheral blood lymphocytes with purified recombinant antigen. These results provide clear evidence that T-cells responsive to islet cell antigens are present in the peripheral blood of type 1 diabetic patients and that these can be recovered for detailed analysis of the T-cell response to islet antigens in the disease.

The T-cell lines were generated by stimulation with *E. coli* expressed recombinant IA-2 followed by expansion of the responding T-cell population with IL-2 and the T-cell mitogen PHA. To establish whether true IA-2 specific T-cells were present, lines that maintained responses to IA-2 after expan-

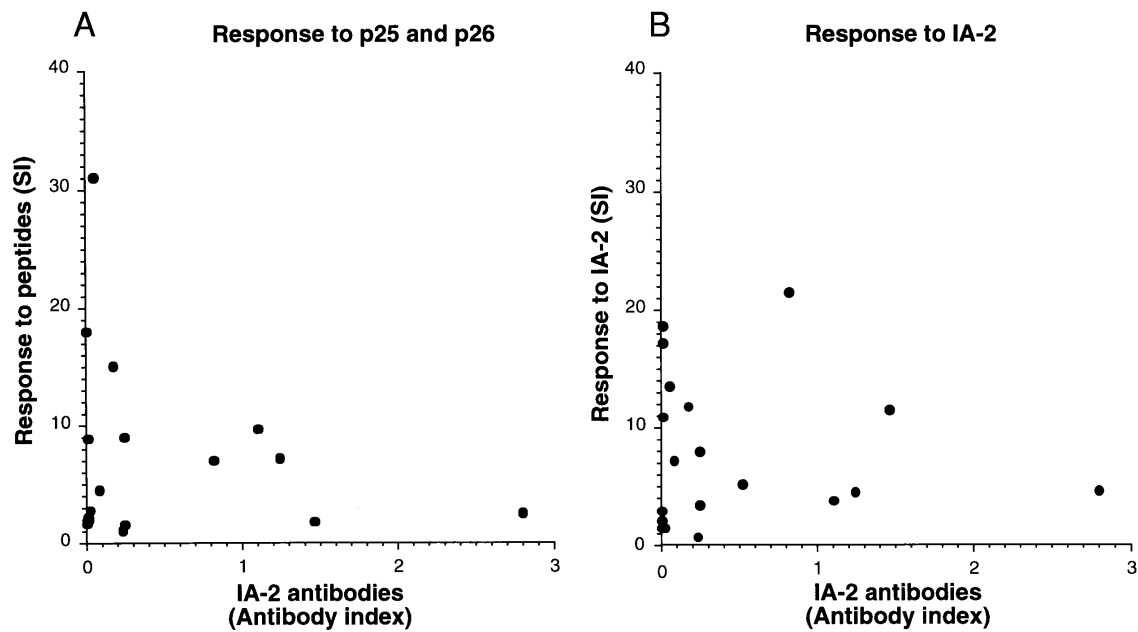


FIG. 5. Relationship between proliferative responses to p25 and p26 peptides (A) or IA-2ic (B) and IA-2 antibody levels in recent-onset type 1 diabetic patients.

sion in culture were tested for reactivity to synthetic peptides representing the entire cytoplasmic domain of IA-2. Two potential T-cell epitopes were identified by this strategy, representing amino acids 751–770 and 831–850 of the IA-2 molecule. To date we have obtained only one line reactive to the former region of IA-2, so the significance of this region as a major T-cell epitope remains to be established. In contrast, T-cell lines from 5 different patients were found to respond strongly to synthetic peptides representing amino acids 831–850 and 841–860, and a line from a healthy control subject responded to a pool of peptides including the 831–850 peptide. The overlapping region (amino acids 841–850) may therefore represent an immunodominant T-cell epitope on IA-2. The 10-amino acid sequence is fully conserved in rat and murine IA-2 and is also present in the human homolog of a closely related PTP (aa 877–886), cloned by several groups and referred to variously as IA-2 $\beta$ , phogrin, or IAR (36,37). The latter protein is also a target for autoantibodies in type 1 diabetes (36,37). Strong primary T-cell proliferative responses to peptides representing this region were detected in the majority of type 1 diabetic patients who had T-cell reactivity to IA-2ic protein. T-cell responses to peptides representing this region have also been reported in at-risk ICA<sup>+</sup> relatives of diabetic patients and in healthy control subjects expressing diabetes-associated HLA alleles (DR4/DQ8 or DR3/DQ2) (38). These results indicate either that this region of IA-2 is exceptionally immunogenic or that it is recognized by T-cells present in the peripheral blood at relatively high precursor frequency. Epitope mapping studies with autoantibodies to IA-2 show that the majority of type 1 diabetic patients have antibodies to epitopes in the region of amino acids 771–979 (9,13,14). This T-cell epitope is therefore located within a highly conserved region that is frequently recognized by autoantibodies of type 1 diabetic patients.

The relatively restricted epitope recognition by IA-2 reactive T-cells from different type 1 diabetic patients contrasts with the considerable heterogeneity in peptide recognition by

T-cell lines generated to GAD in recent studies (34,35). This difference may in part relate to the older ages of patients in these studies (21–54 years), who may therefore be at a later stage of the disease process when the immune response has spread to multiple regions of the molecule. Alternatively, there may be heterogeneity in the MHC class II molecules presenting GAD to the T-cell lines; different GAD-specific T-cells from the same individuals commonly recognize the same epitopes (34,35). However, T-cell lines from different subjects recognizing GAD in the context of the same DR4 molecules were found to recognize different determinants on the molecule (34). Thus, the analysis of T-cell reactivity to GAD in human diabetes does not at present provide evidence for an immunodominant region on the GAD molecule.

Nevertheless, there are precedents for immunodominant regions of autoantigens in human autoimmune disease and in animal models. For example, studies in the NOD mouse model of diabetes have demonstrated a predominant T-cell response to an epitope close to the COOH-terminus of GAD in the early phase of the disease that is followed by spreading of the immune response to other regions of the molecule (39). Similarly, analysis of the T-cell response to proinsulin in HLA-DR4 transgenic mice has implicated an immunodominant region spanning the insulin C-peptide/A-chain boundary (40). In human autoimmune disease, characterization of T-cell lines from patients with multiple sclerosis has identified two immunodominant regions on myelin basic protein (amino acids 84–102 and 143–168) (41,42), of which the former shows degenerate binding to a number of different HLA-DR molecules (43).

As with many studies on the T-cell response to autoantigens in autoimmune disease, it is difficult to ascertain whether the IA-2-reactive T-cells isolated in this study play any role in the disease process. The T-cell line responsive to the 751–770 peptide was found to be restricted by the HLA-DR4 allele that is strongly associated with type 1 diabetes and further analysis of T-cell reactivity to this region



may therefore be warranted. The 751–770 peptide was found to bind with moderate affinity to purified HLA-DR4 in a competitive peptide binding assay, with half-maximal inhibition of binding of an indicator peptide at 7  $\mu\text{mol/l}$  (J. Dromey, M.R.C., unpublished observations). This moderate binding affinity was confirmed using a closely related peptide in another study of IA-2 peptide binding to HLA-DR4 (44), as well as by motif searches by computer (32).

The three other IA-2-specific T-cell lines analyzed in detail in this study were all restricted by class II molecules encoded by HLA-DP genes, which is unusual for T-cell lines in autoimmune disease. Some analyses of genetic susceptibility genes within the HLA region do implicate a contribution for DP alleles independent of the more dominant effects of HLA-DQ and -DR (45,46), but a specific role for the restricting allele, HLA-DP\*0401, has not been reported. Although it has been suggested that the HLA region confers susceptibility to diabetes through the preferential presentation of autoantigen-derived peptides to T-cells, the association of genes within this region with disease is complex and the actual mechanisms involved are far from clear. The HLA-DP restriction of the lines generated in this study does not therefore preclude a role for these T-cells in the disease process. Furthermore, primary proliferative responses of T-cells to synthetic peptides representing amino acids 831–860 of the IA-2 molecule were observed in a high proportion of diabetic patients and their siblings, not all of whom expressed the DP\*0401 allele, indicating that T-cells capable of recognizing this region of IA-2 in the context of other MHC class II molecules are present in the circulation. Interestingly, the immunodominant region of IA-2 that we identified bears regions with high binding affinity to HLA-DR4.

A number of T-cell lines generated in our study failed to respond to IA-2 peptide pools, despite strong responses to the IA-2ic preparation. The failure to respond to the peptides could potentially be a consequence of competitive effects of peptides for MHC class II binding within a particular peptide pool, or of unsuitable choice of peptide dose. The possibility that competition between different HLA-binding peptides within a pool may obscure T-cell reactivity to certain peptides, thus yielding “false-negative” results, has previously been shown to be highly unlikely in practice (33,48). Studies using peptide libraries that were dedicated to bind to particular HLA-restriction elements were successful in identifying T-cell peptide epitopes. Thus, even a millionfold excess of nonepitope peptides, that by design were HLA-binders, was ineffective in inhibiting the response of T-cells to their peptide epitope. Furthermore, in this study the peptide nonresponsive lines were found to respond to a control recombinant purification tag protein expressed in *E. coli* and purified in a similar manner to the IA-2ic preparation. This result implies that the T-cell lines are reacting either to the purification tag sequence or to bacterial contaminants within the recombinant protein preparations. Other studies investigating T-cell reactivity to recombinant autoantigens in autoimmune disorders have reported reactivity to trace bacterial contaminants within recombinant antigen preparations (47,49). The problem of T-cell reactivity to contaminants of antigen preparations is not restricted to bacterially expressed antigen, since T-cell lines generated to purified GAD expressed in a baculovirus system also responded to trace quantities of insect cell or baculovirus proteins (35). The availability of T-cell lines reactive to such components will facilitate

attempts to obtain highly purified preparations of autoantigens that are free of such contaminants. Such reagents will be essential for reliable analysis of autoantigen-specific T-cell responses in type 1 diabetes and other autoimmune disorders.

A relatively high proportion (47%) of recent-onset diabetic patients elicited primary T-cell responses to two synthetic peptides representing amino acids 831–860 of IA-2, consistent with this region having potent immunostimulatory properties. Responses to these peptides were considerably higher than responses to peptides of insulin and GAD that have previously been suggested to be important in the disease. However, nondiabetic siblings also showed positive T-cell responses to the IA-2 peptides. The lack of correlation of T-cell responses to IA-2 and peptides p25 and p26 may be caused by the possibility that other epitopes can also be recognized, or the response to IA-2 protein was directed against a contaminant. We also noted that a significantly higher proportion of nondiabetic siblings than diabetic patients elicited a T-cell response to peptides in the absence of a response to the IA-2ic protein. This observation might imply that there are differences in the efficiency of the processing of IA-2 to generate appropriate peptides, although the potential reactivity to other epitopes of IA-2 or to contaminants in the IA-2ic preparation makes interpretation difficult. Alternatively, the T-cell response to naturally processed IA-2 protein may be regulated in nondiabetic subjects. In other autoimmune diseases it has also proved difficult to clearly demonstrate a disease-specific association between T-cell responses to autoantigens; for example, the frequencies of myelin basic protein-specific T-cells are similar in patients with multiple sclerosis and normal subjects (50). However, subsequent studies indicate that the frequency of myelin basic protein-specific T-cells that are activated is higher in multiple sclerosis compared with healthy subjects (51,52). A recent study (53) suggests that T-cells from type 1 diabetic patients responsive to islet antigens (insulin or  $\beta$ -cell membranes) express markers of a recently activated T-cell population (CD45RA<sup>+</sup>RO<sup>+</sup>). It would therefore be of value to compare the activation state of IA-2-responsive T-cells in diabetic and nondiabetic subjects to assess whether patients differ from control subjects with respect to the subset of lymphocytes that respond to the IA-2 autoantigen (e.g., CD45RA<sup>+</sup>RO<sup>+</sup> in vivo activated T-cells in patients versus CD45RO<sup>+</sup> resting memory T-cells in nondiabetic control subjects).

In summary, we demonstrate that circulating T-cells present in the peripheral blood of patients with type 1 diabetes may preferentially recognize a region of IA-2 between amino acids 831–860 of the molecule. The identification of an immunodominant region of an islet autoantigen could be valuable for the design of procedures to block autoimmune responses to islet antigens as a therapy for type 1 diabetes.

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