TABLE 2
Summary of the clinical characteristics of IAS monoclonal responders at onset of IAS

Ethnic background	Patient no.	Age (years)	Sex	T-IRI (pmol/l × 10^3)	^{125}I-insulin binding (%)	Drug	Associated disease	HLA-DR
Japanese	51	62	M	6.9	52	—	—	4, 8
Norwegian	1	42	M	42.0	60	—	—	4, —
Swiss	1	55	F	1.1	49	CBZ	Graves'	1, 2
Italian	1	57	F	3.7	72	—	—	15, —
Italian	2	5	M	7.2	87	—	—	7, 15
Italian	3	79	M	4.0	64	—	Malaria	4, 11

Carbimazole (CBZ) was administered orally for treatment of Graves' disease. *The drug for malaria was not identified.

binding to human insulin (13) and the diversity of the ratio of κ-to-λ light chain (26).

HLA serological typing and DNA typing. All of the IAS patients were reanalyzed in terms of nucleotide sequences of HLA class II genes. HLA specificities and alleles were described according to Bodmer et al. (27). Serological typing for HLA antigens was performed by the standard microlymphocyte toxicity test (28). Genomic DNA was extracted from the patients, and the nucleotide sequences of HLA class II genes were analyzed by the polymerase chain reaction (PCR)–sequence specific oligonucleotide (29) and PCR–single-strand conformation polymorphism (30) methods and by direct sequencing after DR4-specific PCR amplification.

Statistical analysis. Comparison of incidences was made by the χ^2 calculation, with Yates' correction when necessary.

RESULTS

Two groups defined by clonality. Japanese patients 1 to 50, Korean patients 1 and 2, the Chinese patient, and the white American patient had polyclonal IAAs, whereas Japanese patient 51 and Italian patient 3 showed a single binding affinity by Scatchard analysis (Fig. 1). The Norwegian (18,19) and Swiss (20) patients, as well as Italian patients 1 (21) and 2 (22,23), were previously found to have developed monoclonal autoantibodies to insulin. Therefore, it is likely that the incidence of polyclonal IAS is relatively high among East Asians, whereas monoclonal IAS is more prevalent in Caucasians.

Critical amino acids for IAS polyclonal responders. We have previously shown that Japanese patients 1 to 32, both Korean patients, and the Chinese patient were positive for HLA-DR4/DQ3 (5,6). We have now shown that an additional 16 Japanese IAS polyclonal responders possessed HLA-DR4/DQ3, whereas the remaining 2 (patients 45 and 49) possessed DR9/DQ3 and not DR4; the white American polyclonal responder possessed DR4/DQ3 (Table 1).

The 48 DR4-positive Japanese IAS polyclonal responders comprised 42 DRB1*0406-positive, five DRB1*0403-positive, and one DRB1*0407-positive patient (Table 3). All 48 DR4-positive Japanese IAS polyclonal responders possessed DQA1*0301/DQB1*0302 regardless of the differences in DR4 alleles. The two Korean and the Chinese IAS polyclonal responders were also positive for DRB1*0406/DQA1*0301/DQB1*0302. The phenotype of the white American polyclonal responder was DRB1*0407/DQA1*0301/DQB1*0301. Thus, the DR4-positive IAS polyclonal responders possessed DRB1*0406, DRB1*0403, or DRB1*0407 for DR4 alleles and DQA1*0301/DQB1*0302 or DQA1*0301/DQB1*0301 for DQ3 alleles.

The differences in DQB1 alleles enclosing DQ3 among the IAS polyclonal responders suggest that DQ α- and β-chains are not important in the development of IAS. We recently showed that T-cells from polyclonal Japanese IAS patients with DRB1*0406/DQA1*0301/DQB1*0302 alleles proliferated in the presence of autologous antigen-presenting cells that had been exposed to 40 μmol/l human insulin (6). The proliferative response of T-cells from Japanese IAS patients was completely blocked by anti-HLA-DR but not by anti-HLA-DQ monoclonal antibodies (7). Moreover, experiments with DRB1*0406 transfectants supported the view that DR gene products participate in the presentation of human insulin antigens (7).

The HLA-DR β1-chains encoded by DRB1*0406, DRB1*0403, and DRB1*0407 share a sequence motif (Leu-Leu-Glu-Gln-Arg-Arg-Ala-Glu) that spans the amino acid residues 67–74 of the third hypervariable region. The two DR9/DQ3 Japanese

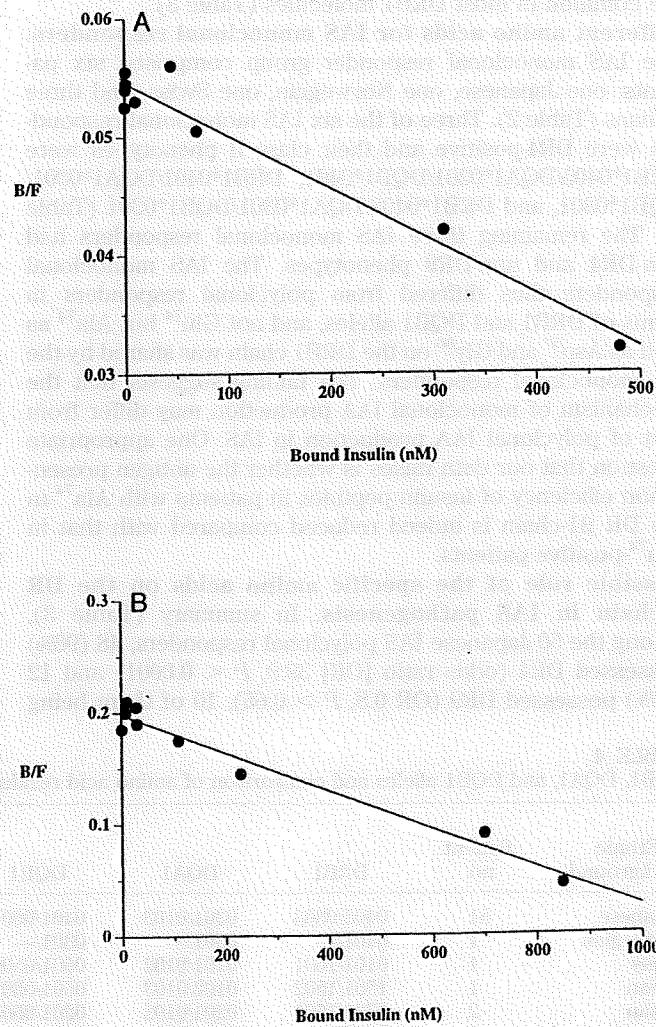

FIG. 1. Scatchard analysis of IAA using ^{125}I-labeled A14-human insulin. Serum deinsulinized by dextran-coated charcoal (35) was used for the Scatchard analysis (36). *A:* Japanese patient 51. *B:* Italian patient 3.

TABLE 3
Incidence of DRB1 alleles, Glu^{74} in DR β1-chain and DBQ1 alleles in Japanese IAS polyclonal responders and control subjects

	IAS patients	Control	OR (95% confidence interval)	DRB1 chain amino acid residue		
				37	74	86
n	50	106	—	—	—	—
DRB1 allele						
DR4	48 (96)	40 (38)	39.6 (9.12–171)	—	—	—
DR9	12 (24)	29 (27)	0.8 (0.39–1.82)	Asn	Glu	Gly
DRB1*0406	42 (84)	9 (8)	56.6 (20.4–156)	Ser	Glu	Val
DRB1*0403	5 (10)	7 (7)	1.6 (0.47–5.22)	Tyr	Glu	Val
DRB1*0407	1 (2)	2 (2)	1.1 (0.09–12.0)	Tyr	Glu	Gly
Glu^{74} in β-chain	50 (100)	70 (66)	52.3 (6.95–393)	—	—	—
DQB1 allele						
DQA1*0301	50 (100)	74 (70)	44.1 (5.84–332)	—	—	—
DQB1*0302	48 (96)	26 (25)	73.8 (16.8–325)	—	—	—
DQA1*0301/DQB1*0302	48 (96)	23 (22)	86.6 (18.8–380)	—	—	—

Data are *n* (%) or OR (95% confidence interval).

IAS polyclonal responders (patients 45 and 49) were DRB1*0901/DQA1*0301/DQB1*0303 homozygotes. The products of DRB1*0406, DRB1*0403, DRB1*0407, and DRB1*0901 share the sequence motif Arg-Arg-Ala-Glu, corresponding to amino acids 71–74 of the DR β1-chain. Comparison of this region of the DRB1 chain and other DRB1 allele products reveals that Arg^{71} and especially Glu^{74} may be important for polyclonal IAA production in IAS, whereas residues 72 and 73 (Arg-Ala) are common in most DRB1 molecules (Table 3).

Different amino acids for IAS monoclonal responders. The IAS monoclonal responder group comprised six patients: one Japanese, one Norwegian, one Swiss, and three Italians (Table 2). Three of the six IAS monoclonal responders were DR4-positive and their class II phenotypes were DRB1*0405/DQA1*0301/DQB1*0401, DRB1*0401/DQA1*0301/DQB1*0301, and DRB1*0402/DQA1*0301/DQB1*0301 (Table 4). The remaining three IAS monoclonal responders had non-DR4 and non-DR9 phenotypes. The IAS monoclonal responders thus differed from polyclonal responders in terms of DRB1 and DQB1 alleles, and not Glu^{74} but Ala^{74} as well as Asp^{57} and Gly^{86} on the DRB1 chain was shared by the six monoclonal responders. The finding suggests that the mechanism of monoclonal IAA production may differ from that of polyclonal IAA production in IAS. One appropriate question that our data raises is whether the antigen presentation efficiency of insulin peptides in patients with Ala^{74} in the DR β1-chain is indeed reduced compared with that in Glu^{74}-positive patients.

Possible role of the specific amino acids on the DR β-chain in IAS pathogenesis. In summary (Table 3), among the 50 Japanese IAS polyclonal responders, 48 (96%) possessed DR4 (odds ratio [OR] 39.9, $P < 0.0001$) and 12 (24%) possessed DR9 (OR 0.8, $P > 0.65$), 10 of them being DR4/9 heterozygotes and two being DR9 homozygotes. Among the DR4-positive patients, 42 (84% of polyclonal responders) had DRB1*0406 (OR 56.6, $P < 10^{-6}$), while five (10% of polyclonal responders) had DRB1*0403 (OR 1.6, $P > 0.4$), and one (2% of polyclonal responders) had DRB1*0407 (OR 1.1, $P > 0.96$). It is of great interest that DRB1*0406 and DRB1*0403 products differ by only a single amino acid: Ser^{37} for DRB1*0406 and Tyr^{37} for DRB1*0403. Table 3 also shows the percentage of Glu^{74} in the DR β-chain (OR 52.3, $P < 10^{-6}$). The presence of Glu^{74} in all DR β-chains may be related to the development of IAS as well, although the development of IAS presumes the presence of HLA-DR4. On the other hand, DQA1*0301, DQB1*0302, and DQA1*0301/DQB1*0302 exhibited higher ORs (Table 3). However, it is difficult to determine the primary susceptibility gene(s) from these ORs, since there are no significant differences among these values. Moreover, the presence of DRB1*0301 in a white American IAS patient, blocking experiments of T-cell proliferative response by monoclonal anti-DR and anti-DQ antibodies (7), and T-cell proliferation experiments using DRB1*0406 transfectants as antigen-presenting molecules (7) support the hypothesis that DR rather than DQ gene products participate in the presentation of human insulin.

DISCUSSION

Based on the findings described above, we conclude that *1*) DR4 is the dominant phenotype in terms of susceptibility to IAS, *2*) DRB1*0406 is associated with the highest risk for the susceptibility to IAS, and *3*) Glu^{74} in the DR4B1 chain is essential for polyclonal IAA production in IAS. Ser^{37} in the DR4 β1-chain may have a significant additive effect on polyclonal autoantibody production (Table 3).

TABLE 4
DRB1, DQA1, and DQB1 alleles and comparison of amino acid residues in the DR β1 chain in IAS monoclonal responders

Ethnic background	Patient no.	DRB1	DQA1	DQB1	DRB1 chain amino acid residue				
					37	47	57	74	86
Japanese	51	0405/0803	0301/0103	0401/0601	Tyr	Tyr	Ser/Asp	Ala/leu	Gly/Val
Norwegian	1	0401/—	0301/—	0301/—	Tyr	Tyr	Asp	Ala	Gly
Swiss	1	0101/1601	0101/0102	0501/0502	Ser	Tyr	Asp	Ala	Gly
Italian	1	1501/1502	0102/0103	0601/0602	Ser	Phe	Asp	Ala	Gly/Val
Italian	2	0701/1501	0201/0102	0201/0602	Ser/Phe	Phe/Tyr	Val/Asp	Ala/Gln	Gly/Val
Italian →	3	0402/1101	0301/0501	0301/0302	Tyr	Phe/Tyr	Asp	Ala	Gly

Karr et al. (31) showed that a $Glu^{74} \rightarrow Ala^{74}$ substitution in the DR7 β1-chain abolished the ability of the DR7 molecules to present peptides to specific T-cell clones or permitted minimal presentation only at the highest peptide concentration, indicating that residue 74 is critical for recognition of the peptide-DR7 complex. Olson et al. (32) also showed the importance of the third hypervariable region in DR1 molecules for DR1-restricted recognition of a pertussis toxin peptide; an $Ala^{74} \rightarrow Glu^{74}$ substitution abrogated recognition by the antigen-specific T-cell clones. The strict requirement for Glu^{74} in the third hypervariable region of the DR4 β1-chain for the development of polyclonal IAAs suggests that this charged residue serves in the effective presentation of a specific insulin-derived peptide to specific T-cells.

The three-dimensional structure of the HLA class II DR1 molecules determined by X-ray crystallography has shown an open-ended groove, in which the peptides processed by antigen-presenting cells are bound as straight extended chains (33) and an anchoring peptide side chain of the processed peptides was found to fit in a prominent nonpolar pocket near one end of the binding groove (33). Recently, Matsushita et al. (34) reported that a peptide of human insulin α-chain (^{8}TSICSLYQLE^{17}) was shown to bind specifically to DRB1*0406 using its ^{10}IxxLxQ^{15} motif. The second anchor residue was reported to exhibit allele specificity in binding, especially with the amino acid residue 74 of DRB1 chain (34). However, the interaction of L (Leu) of insulin peptide with ^{74}Glu in the DR4 β-chain has remained questionable because Leu was a hydrophobic residue and Glu was an acidic residue.

Because polyclonal IAS patients exhibit typical polyclonal immunoglobulin G response to human insulin, the response may be an antigen-driven immune one with T-cell help. Accordingly, typical HLA- and peptide-restricted recognition may contribute to the initiating event in the pathogenesis of IAS, in which Glu^{74} may act as the primary residue in the peptide-binding interaction.

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Patients with Graves' Disease who Developed Insulin Autoimmune Syndrome (Hirata Disease) Possess HLA-Bw62/Cw4/DR4 Carrying DRB1*0406

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ABSTRACT

The insulin autoimmune syndrome (IAS) is characterized by the following diagnostic criteria: severe spontaneous hypoglycemia without evidence of exogenous insulin administration, high levels of total serum immunoreactive insulin, and the presence of a high titer of antiinsulin antibody. Just before the onset of IAS, 13 of the 35 (37%) patients with IAS examined in this study had taken methimazole for the treatment of Graves' disease. To investigate the difference between the Graves' disease patients treated with methimazole who developed IAS and other IAS patients, HLA class II genes in both groups were analyzed by serological and DNA typing methods. All 13 patients with Graves' disease who developed IAS possessed a specific allelic combination, Bw62/Cw4/DR4 carrying DRB1*0406, whereas only 1 of 50 Graves' disease patients without IAS had Bw62/Cw4/DR4 (odds ratio, 891; $P < 1 \times 10^{-10}$) and carried not DRB1*0406 (odds ratio, 2727; $P < 1 \times 10^{-10}$), but DRB1*0405. Of the 22 IAS patients without Graves' disease, 13 had the combination Bw62/Cw4/DR4 carrying DRB1*0406 (odds ratio, 19.0; $P < 0.07$). Thus, it is highly likely that patients with Graves' disease develop IAS via treatment with methimazole when their Bw62/Cw4/DR4 carry DRB1*0406. (*J Clin Endocrinol Metab* 77: 249–254, 1993)

THE FIRST report of spontaneous hypoglycemia without evidence of exogenous insulin administration was published by Hirata *et al.* in 1970 (1). The combination of high levels of total serum immunoreactive insulin, the presence of insulin autoantibodies, and fasting hypoglycemia has been termed insulin autoimmune syndrome (IAS) and is reported to be the third leading cause of hypoglycemia in Japan (2). The first case of spontaneous hypoglycemia with insulin autoimmunity in Graves' disease was reported by Hirata *et al.* (3). Burch *et al.* (4) recently reported a black IAS patient and summarized other IAS cases from Japan, some of whom had had Graves' disease treated with methimazole. As Hirata noted in 1983 (5), patients with Graves' disease who had received methimazole had a predisposition to develop IAS. Our recent study of the clinical characteristics of 190 Japanese patients with IAS showed that before the onset, 42% of them had received medication (methimazole, α-mercaptopropionyl glycine, and glutathione, which are all sulfhydryl compounds) (our manuscript in preparation). None of the IAS patients with Graves' disease had received propyonylthiouracil before the onset of IAS. This suggests that sulfhydryl compounds play an important role in the development of IAS. Recently, we found that all IAS patients so far examined exhibited characteristic DNA types, DRB1*0406/DQA1*0301/DQB1*0302 (6). In this study, we examined differences in HLA class II genes between the patients with Graves' disease treated with methimazole who developed IAS and those who had received methimazole, but did not develop IAS.

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Subjects and Methods

Patients

Blood samples from 13 patients with Graves' disease who developed IAS after receiving methimazole and from 22 patients with IAS without Graves' disease were obtained by their primary physicians, and blood samples from 50 patients with Graves' disease receiving methimazole treatment who had not developed IAS were obtained from the Endocrinology Clinic of Tokyo Women's Medical College. The diagnosis of IAS was based on the clinical presentation of 1) spontaneous or fasting hypoglycemia (usually <40 mg/dL) without evidence of insulin injection, and 2) laboratory findings of high levels of total immunoreactive insulin (IRI) and a high titer of insulin antibody in serum, as shown in Tables 1A and 1B. IRI was determined in duplicate by double antibody RIA after extraction with 12.5% Polyethylene Glycol 6000; insulin antibody was determined by a binding assay, using [^{125}I]human insulin (7). The 13 patients with Graves' disease developed IAS within 1.5 months after starting methimazole treatment. The hypoglycemic attacks disappeared gradually in approximately 1 week after they ceased taking methimazole (8, 9). Although the 50 Graves' disease patients without IAS had been taking methimazole for more than 6 months, they had not had hypoglycemic attacks. There was no difference in the hypoglycemic attacks between the IAS patients with and without Graves' disease. Both groups exhibited trembling and numbness of the fingers and blood glucose levels below 40 mg/mL. IRI levels and [^{125}I]insulin binding did not differ in the two groups (Tables 1A and 1B). Insulinoma was sought by computed tomography, ultrasonography, angiography, and pancreaticocholangiography, but none of the patients with IAS had insulinoma. All have been healthy since the resolution of the hypoglycemic attacks. Fifty-one healthy Japanese subjects served as controls.

TABLE 1A. Clinical summary of IAS patients with Graves' disease at the onset of IAS

Patient no.	Age (yr)	Sex	IRI (pmol/L)	[^{125}I]Insulin binding (%)	Drug	Associated disease
1	26	F	136,598	69	MTZ	Graves'
2	61	F	14,350	90	MTZ	Graves' Hashimoto' and IgA nephropathy
3	45	F	8,610	32	MTZ	Graves'
4	39	F	3,229	69	MTZ	Graves'
5	18	M	1,322	81	MTZ	Graves', drug-induced arthritis, and dermatitis
6	21	M	56,180	65	MTZ	Graves'
7	36	F	230,245	79	MTZ	Graves'
8	43	F	21,525	57	MTZ	Graves'
9	31	F	10,691	62	MTZ	Graves'
10	54	F	71,750	70	MTZ	Graves'
11	49	F	21,525	83	MTZ	Graves'
12	70	F	55,965	94	MTZ	Graves'
13	18	F	43,574	82	MTZ	Graves'

MTZ, Methimazole. Age was that at the onset of IAS. The methods for the total IRI and [^{125}I]human insulin binding assays used have been described previously (7).

TABLE 1B. Clinical summary of IAS patients without Graves' disease at the onset of IAS

Patient no.	Age (yr)	Sex	IRI (pmol/L)	[^{125}I]Insulin binding (%)	Drug	Associated disease
1	52	F	65,228	73	GTG	Bronchial asthma
2	47	M	27,983	79		
3	52	M	15,929	38		
4	36	M	9,751	59		
5	62	M	15,068	69		
6	54	M	5,310	81	MPG	Liver dysfunction
7	61	F	6,099	72	MPG	Cataract
8	44	M	3,588	70	MPG	Dermatitis
9	69	M	14,666	65		
10	57	F	3,372	51		
11	68	F	14,063	68	MPG	Liver dysfunction
12	64	M	5,446	82	MPG	Dermatitis
13	58	F	14,350	81		
14	49	M	14,278	48	MPG	Liver dysfunction
15	69	F	199,896	67	TBM	NIDDM[a]
16	55	M	1,033	48	MPG	Liver dysfunction
17	53	F	4,190	55		
18	68	M	6,458	64	MPG	Liver dysfunction
19	66	F	25,033	57	MPG	Cataract
20	54	M	4,090	67	MPG	Liver dysfunction
21	67	F	143,500	80		
22	50	M	2,088	64	GTT	Urticaria

GTG, Gold thioglucose; MPG, α-mercaptopropionyl glycine; TBM, tolbutamide; GTT, glutathione.

[a] The patient developed IAS after taking 1 tablet (250 mg) of TBM.

HLA serological typing

Serological typing for HLA antigens was performed by the standard microlymphocyte toxicity test (10). HLA specificities and alleles were described according to the Nomenclature for Factors of the HLA System, 1987 (11), and the Nomenclature for Factors of the HLA System, 1990 (12), respectively.

DNA typing by polymerase chain reaction (PCR)-sequence-specific oligonucleotide (SSO)

Genomic DNA was extracted from the Graves' disease patients and control subjects and the second exons of the DRB1, DQA1, and DQB1 genes were amplified with the sets of primers DR4B1AMP-A and DRBAMP-B, GH26 and GH27 (13), and GH28 and GH29 (14), respectively, shown in Table 2, using a *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, CT) (15). Seven DR4-related SSO probes (DRB37–1, DRB70–1, DRB57–1, DRB70–3, DRB70–5, DRB86–1, and DRB86–3) for the DRB1 gene, one SSO probe (DQA52–1) for the DQA1 gene, and five SSO probes (DQB26–2, DQB26–3, DQB37–2, DQB57–7, and DQB70–4) for the DQB1 genes (shown in Table 2), which had all been labeled with digoxigenin-11-deoxy-UTP, were hybridized with slot-blotted PCR samples, and the chemiluminescent signal of 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)phenyl-1,2-dioxetane (Boehringer Mannheim, St. Louis, MO) hydrolysis by alkaline phosphatase-labeled antidigoxigenin antibody (Boehringer Mannheim) was detected using Fuji x-ray film (Fuji X-Ray Co, Kanagawa, Japan) (16).

Statistics

Comparisons of incidences were made by the χ^2 test, with Yates' correction when necessary.

Results

Table 1A shows a clinical summary of the Graves' disease patients with IAS at the onset of the latter condition. They all showed more than 400 μU/mL IRI and a [^{125}I]insulin binding percentage of more than 30%. The 50 patients with Graves' disease who had not developed IAS had less than 72 pmol/L IRI and [^{125}I]human insulin binding of less than 5% (data not shown), which are within the normal ranges. Table 1B shows a clinical summary of the IAS patients without Graves' disease. They all had more than 1033 pmol/L IRI and [^{125}I]human insulin binding of more than 30%.

Table 3 shows the results of serological HLA typing. All 13 patients with Graves' disease who developed IAS had a characteristic antigenic combination of Japanese, Cw4, Bw62, and DR4 (17), whereas only 1 of the 50 patients with Graves' disease who had not developed IAS had this antigenic combination. Table 4 shows the phenotype frequencies of HLA antigens. The incidence of the following antigens was significantly different in the 2 groups: IAS with Graves' disease *vs.* IAS without Graves' disease, Cw4 ($P < 0.007$) and Bw62/Cw4/DR4 ($P < 0.007$); Graves' disease with IAS *vs.* Graves' disease without IAS, Bw62 ($P < 1 \times 10^{-8}$), Cw4 ($P < 1 \times 10^{-10}$), DR4 ($P < 4 \times 10^{-5}$), and Bw62/Cw4/DR4 ($P < 1 \times 10^{-10}$).

We next analyzed the nucleotide sequences of the HLA class II genes by the PCR-SSO method. DR4 group-specific amplification was performed for the DRB1 gene. As shown in Table 5A, a striking deviation in the frequency of a specific DR4 allele (DRB1*0406) was observed. All 13 patients with Graves' disease who developed IAS possessed this allele, which was positive for DRB37–1, DRB70–3, and DRB86–3 and negative for DRB57–1, DRB70–1, DRB70–5, and DRB86–1 (odds ratio, 2727; $P < 1 \times 10^{-10}$). The IAS patients without Graves' disease also had DRB1*0406 (5). Moreover, all 35 IAS patients with and without Graves' disease had DQA1*0301 and DQB1*0302 (odds ratio, 35.9; $P < 6 \times 10^{-4}$; and odds ratio, 1.68; $P < 1 \times 10^{-10}$, respectively). Table 5B shows the allele frequencies of the DR4 group in patients with Graves' disease who had not developed IAS; of the 50 patients in this category, 18 had DR4, which consisted of 2

TABLE 2. Oligonucleotides panel

HLA gene	Primers	Nucleotide sequences
DRB1	DR4B1AMP-A	5′-GTTTCTTGGAGCAGGTTAAAC-3′
	DRBAMP-B	5′-GCCGCTGCACTGTGAAGCTCT-3′
DQA	GH26	5′-GTGCTGCAGGTGTAAACTTGTACCAG-3′
	GH27	5′-GACGGATCCGGTAGCAGCGGTAGAGTTG-3′
DQB	GH28	5′-CTCGGATCCGCATGTGCTACTTCACCAACG-3′
	GH29	5′-GTGCTGCAGGTAGTTGTTGTGTCTGCACAC-3′

Probes	Nucleotide sequences	Amino acid positions
DRB37-1	5′-CAAGAGGAAGTCCGTGCGCT-3′	^{34}QEESVR
DRB57-1	5′-CGGCCTAGCGCCGAGTAC-3′	^{56}RPSAEY
DRB70-1	5′-GCAGAGGCGGGCCGCGGT-3′	^{70}QRRAAV
DRB70-3	5′-GCAGAGGCGGGCCGAGGT-3′	^{70}QRRAEV
DRB70-5	5′-ACCGCGGCCCGCTTCTGC-3′	^{70}QKRAAV
DRB86-1	5′-AACTACGGGGTTGGTGAG-3′	^{82}NYGVGE
DRB86-3	5′-AACTACGGGGTTGTGGAG-3′	^{82}NYGVVE
DQA52-1	5′-TTCCGCAGATTTAGAAGAT-3	^{51}FRRSRR
DQB26-2	5′-CGTTATGTGACCAGATACA-3′	^{25}RYVTRY
DQB26-3	5′-CGTCTTGTGACCAGATACA-3′	^{25}RLVTRY
DQB37-2	5′-GAGAGGAGTACGCACGCTTC-3′	^{35}EEYARFD
DQB57-7	5′-GGCCGCCTGCCGCCGAGTAC-3′	^{54}GPPAAEY
DQB70-4	5′-GGACCCGGGCGGAGTTGGA-3′	^{71}TRAELD

TABLE 3. Serological HLA typing of patients with IAS

Patient no.	A	B	C	DR	DQ
A) IAS with Graves' disease					
1	2,26	40,w62	w4,-	4,-	NT
2	11,24	w62,-	w4,-	4,-	w3,-
3	11,-	w52,w62	w1,w4	4,w8	w1,w3
4	24,26	w62,-	w4,-	4,-	NT
5	24,-	w54,w62	w1,w4	4,-	w3,-
6	2,26	35,w62	w4,-	4,12	NT
7	24,26	35,w62	w3,w4	2,4	w1,w3
8	2,11	w60,w62	w3,w4	4,-	NT
9	2,26	39,w62	w4,w7	4,9	w3,-
10	2,26	w61,w62	w4,-	4,-	w3,-
11	24,31	w54,w62	w1,w4	4,-	w3,w4
12	11,24	w54,w62	w4,-	4,-	w3,w4
13	2,11	w46,w62	w1,w4	4,-	w3,-
B) IAS without Graves' disease					
1	11,33	44,w62	w4,-	4,w13	w1,w3
2	11,24	44,w62	w4,-	4,9	NT
3	24,-	w52,w62	w1,w3	4,w6	NT
4	11,24	w60,w62	w4,-	4,9	NT
5	11,33	44,w62	w4,-	4,9	w3,-
6	11,31	16,w62	w4,w7	2,4	NT
7	24,26	35,w61	w3,-	4,9	NT
8	24,26	w62,-	w3,w4	2,4	NT
9	24,w33	17,w62	w3,w4	4,w6	w1,s3
10	2,11	w61,w62	w4,-	4,9	w3,-
11	26,-	35,-	w3,-	4,w8	w3,-
12	2,33	44,35	w3,-	4,w13	NT
13	11,24	w61,w62	w4,-	4,w12	w3,w7
14	24,31	w62,-	w3,-	4,w12	w3,w7
15	2,11	51,w62	w4,-	4,-	w3,-
16	2,-	w46,-	w11,-	4,w8	w1,w3
17	2,24	15,35	w3,-	4,9	w3,-
18	2,11	w55,w62	w1,w3	4,w6	w1,w3
19	11,-	w62,-	w1,w3	4,9	NT
20	11,26	35,w62	w3,w4	4,-	w3,w4
21	2,11	w62,-	w4,-	4,w8	w1,w3
22	11,31	7,w62	w4,w7	1,4	NT

NT, Not tested.

TABLE 4. Phenotype frequencies of HLA antigens

Antigen	IAS with Graves' (n = 13)	IAS without Graves' (n = 22)	Odds ratio (95% confidence interval)	*P*
A) IAS with and without Graves' disease				
All	4 (31)[a]	13 (59)[a]	0.31 (0072–1.32)	<0.11
A24	5 (38)	8 (36)	1.09 (0.27–4.50)	<0.91
B35	2 (15)	5 (23)	0.62 (0.10–3.77)	<0.60
Bw62	13 (100)	16 (73)	7.36 (0.7–70.6)	<0.04
Cw4	13 (100)	13 (59)	19.0 (2.14–169)	<0.007
DR1	0 (0)	1 (4)	0.53 (0.04–6.42)	<0.44
DR2	1 (8)	2 (9)	0.83 (0.06–10.2)	<0.89
DR4	13 (100)	22 (100)	0.60 (0.03–10.3)	1.0
DRw6/w13/w14	0 (0)	5 (22)	0.12 (0.01–1.10)	<0.06
DRw8	1 (8)	3 (14)	0.53 (0.05–5.68)	<0.06
DR9	1 (8)	8 (36)	0.15 (0.01–1.34)	<0.07
DRw12	1 (8)	2 (9)	0.83 (0.06–10.2)	<0.89
Bw62/Cw4/DR4	13 (100)	13 (59)	19.0 (2.14–169)	<0.007
	Graves' with IAS (n = 13)	Graves' without IAS (n = 50)	Odds ratio (95% confidence interval)	
B) Graves' disease with and without IAS				
All	4 (31)[a]	14 (28)[a]	1.14 (0.30–4.32)	<0.85
A24	5 (38)	25 (50)	0.63 (0.18–2.18)	<0.46
B35	2 (15)	9 (18)	0.83 (0.16–4.41)	<0.83
Bw62	13 (100)	8 (16)	135 (15.6–1160)	$<1 \times 10^{-8}$
Cw4	13 (100)	5 (10)	223 (24.8–2015)	$<2 \times 10^{-10}$
DR1	0 (0)	0 (0)	3.74 (0.22–63.7)	1.0
DR2	1 (7)	0 (0)	12.1 (1.20–144)	<0.05
DR4	13 (100)	18 (36)	47.4 (5.78–389)	$<4 \times 10^{-5}$
DRw8	1 (7)	15 (30)	0.19 (0.02–1.63)	<0.10
DR9	1 (7)	20 (40)	0.12 (0.01–1.03)	<0.03
DRw12	1 (7)	4 (8)	0.95 (0.09–9.38)	<0.97
DRw13	0 (0)	7 (14)	0.21 (0.02–1.87)	<0.16
DRw14	0 (0)	2 (4)	0.72 (0.06–1.45)	<0.47
DRw15	0 (0)	19 (38)	0.06 (0.01–0.49)	<0.008
Bw62/Cw4/DR4	13 (100)	1 (2)	891 (75.1–10,560)	$<1 \times 10^{-10}$

The number of subjects is given, with the percentage in parentheses.

TABLE 5A. Allele frequencies of DR4 group in patients with Graves' disease

DR4 allele	Graves' with IAS (n = 13)	Graves' without IAS (n = 50)	Odds ratio (95% confidence interval)	*P*
04	13 (100)[a]	18 (36)[a]	47.4 (5.78–389)	$<4 \times 10^{-5}$
0401	0 (0)	0 (0)	3.74 (0.22–63.6)	1.0
0403	0 (0)	2 (4)	0.72 (0.06–7.45)	<0.47
0405	0 (0)	14 (28)	0.09 (0.01–0.77)	<0.034
0406	13 (100)	0 (0)	2,727 (160–46,400)	$<1 \times 10^{-10}$
0407	0 (0)	1 (2)	1.22 (0.10–14.4)	<0.61

All 22 patients with IAS without Graves' disease also possessed DRB1*0406.
[a] The number of subjects is given, with the percentage in parentheses.

with DRB1*0403, 14 with DRB1*0405, 1 with DRB1*0407, and 1 with DRB1*0408. That is, none of the patients with Graves' disease without IAS had DRB1*0406. A significant difference was observed only in the DRB1*0406 frequency ($P < 0.002$). DRB1*0406 was reduced in the patients with Graves' disease without IAS. No other remarkable difference was found.

Discussion

In this study, we showed differences in HLA antigen frequency in Graves' disease patients who developed IAS and in those patients who had not developed this condition, despite receiving the same treatment (methimazole). All 13 Graves' disease patients who developed IAS had Cw4, Bw62, and DR4, whereas 13 of the 22 IAS patients without Graves' disease had Cw4, Bw62, and DR4 (odds ratio, 19.0; $P < 0.007$). Regarding class II, all of the 35 IAS patients with and without Graves' disease had DR4 (DRB1*0406). On the other hand, only 1 of the 50 Graves' disease patients who had not developed IAS had the combination of Cw4, Bw62, and DR4, with the DNA typing revealing a different subtype, DRB1*0405; none of the Graves' disease patients had

TABLE 5B. Allele frequencies of DR4 group in patients with Graves' disease who did not develop IAS

DR4 allele	Graves' without IAS (n = 50)	Control (n = 51)	Odds ratio (95% confidence interval)	P
04	18 (36)[a]	20 (39)[a]	0.87 (0.38–1.95)	<0.7
0401	0 (0)	1 (2)	0.33 (0.02–3710)	1.0
0403	2 (4)	6 (12)	0.31 (0.06–1.62)	<0.2
0405	14 (28)	9 (18)	1.81 (0.70–4.68)	<0.2
0406	0 (0)	7 (14)	0.06 (0.07–0.47)	<0.002
0407	1 (2)	0 (0)	3.06 (0.26–34.8)	<0.9
0408	1 (2)	0 (0)	3.06 (0.26–34.8)	<0.9

[a] The number of subjects is given, with the percentage in parentheses.

DRB1*0406/DQA1*0301/DQB1*0302. The development of IAS in Graves' disease could be divided into 2 groups on the basis of HLA haplotypes: a Bw62/Cw4/DR4 (DRB1*0406) group and a group not showing these characteristics.

To date, the literature worldwide regarding the association of Graves' disease with HLA antigens is voluminous. The association of Graves' disease in Japanese subjects with HLA-Dw12 (DRw15) was previously reported by Sasazuki *et al.* (18). Our present findings showed that 19 (38%) of 50 patients with Graves' disease had DRw15 compared with 8 (16%) of 51 healthy controls ($P < 0.05$). There were no other differences between this group with Graves' disease without IAS and healthy Japanese controls. However, all 13 patients with Graves' disease who developed IAS had a particular combination of Cw4, Bw62, and DR4 (DRB1*0406), while only 59% of the 22 non-Graves' disease patients with IAS exhibited this combination. This finding suggests that the combination of Cw4, Bw62, and DR4 carrying DRB1*0406 is necessary, but not sufficient, to cause the development of IAS when Graves' disease patients have received methimazole.

Although Bw62/Cw4/DR4 is a particular HLA haplotype combination in the Japanese, it is possible that in IAS patients with Graves' disease, class I molecules might help class II molecules present processed insulin on antigen-presenting cells (APC). According to the clinical findings of 190 patients with IAS in Japan (our manuscript in preparation), we found that 42% of the IAS patients had been taking medication before the onset on the condition; methimazole (MTZ), α-mercaptopropionyl glycine, and glutathione had been taken. They are all sulfhydryl compounds. In this present study, all 13 methimazole-treated Graves' disease patients with IAS had Bw62/Cw4/DR4 (DRB1*0406), whereas 4 of 11 non-Graves' IAS patients who had been taking α-mercaptopropionyl glycine and glutathione had Bw62/Cw4/DR4 (DRB1*0406). It is possible that there might be an epistatic effect between class I and class II molecules on sulfhydryl compounds that brings about the development of IAS in Graves' disease patients.

One important function of the endocytic pathway in APC is to alter the structure of protein antigens so that they may interact with class II histocompatibility glycoproteins. To maintain antigenicity, the minimal length disulfide bond-containing peptides of insulin still require processing by the operational definition. Naquet *et al.* (19) described a minimal determinant comprised of insulin residues A (1–14) disulfide linked to insulin-B (7–15). Further, Jensen (20) demonstrated that this requirement can be bypassed by incubating insulin with reducing agents in the presence of aldehyde-fixed APC. Methimazole, a sulfhydryl compound, may play a role as a reducing agent, cleaving the disulfide bonds of insulin. In the presence of methimazole, human insulin could easily be presented and recognized in the context of the DRB1*0406 gene product on the APC of Graves' patients. A patient with Graves' disease who possesses Bw62/Cw4/DR4 carrying DRB1*0406 may be at risk of developing IAS when methimazole is administered.

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Recognition of Human Insulin in the Context of HLA-DRB1*0406 Products by T Cells of Insulin Autoimmune Syndrome Patients and Healthy Donors

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ABSTRACT. Our recent study indicated that all the insulin autoimmune syndrome (IAS) patients had specific HLA class II alleles, the DRB1*0406, DQA1*0301, and DQB1*0302, which allowed T cells to proliferate when autologous APC were exposed to human insulin. The study implied that gene products of DRB1*0406, DQA1*0301, and/or DQB1*0302 may be involved in the presentation of human insulin to T cells. We therefore examined T cell response of healthy donors with different HLA phenotypes to human insulin using an autologous MLR system. The T cells from not only IAS patients but also healthy donors were able to proliferate after exposure of human insulin to autologous APC with DRB1*0406, DQA1*0301, and DQB1*0302 products. The class II molecules are considered to be involved in the recognition of human insulin by T cells. The proliferative response of T cells was completely blocked by anti-HLA-DR mAb and not by anti-HLA-DQ mAb or other mAb. Furthermore, human insulin-specific CD4-positive T cell clones were established from blast cells in autologous MLR of PBMC from two healthy donors with DRB1*0406 in the presence of human insulin. Using DRB1*0406-transfected L cells as APC, we confirmed that these T cell clones recognize human insulin in the context of gene products of DRB1*0406. These results provide the first evidence that HLA-DRB1*0406 products act as the dominant restriction element for the presentation of human insulin to T cells, and suggests that this particular class II gene, HLA-DRB1*0406, contributes to the development of IAS. *Journal of Immunology,* 1993, 151: 5770.

In the animal model, insulin has been studied extensively regarding the genetic and cellular regulation of T cell recognition in class II MHC-restricted cell-mediated immune responses (1, 2). In contrast, in humans, the class II MHC restriction element in the presentation of insulin to T cells was not clearly identified, although some HLA-DR molecules have been shown to act as dominant restriction elements for insulin-specific T cell lines or T cell clones (3, 4).

Recently, we showed that T cells from IAS[2] patients with HLA-DRB1*0406, DQA1*0301, and DQB1*0302 alleles proliferated in response to human insulin only by exposing the autologous APC to human insulin (5). Hence, we examined whether T cells from healthy donors with DRB1*0406, DQA1*0301, and DQB1*0302 could also proliferate by exposure of human insulin to APC, and

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[2] Abbreviations used in this paper: IAS, insulin autoimmune syndrome; DTT, dithiothreitol; SI, stimulation index.

whether the proliferative response of T cells to human insulin is restricted to the products of these genes.

In this study we have focused on the recognition of human insulin by T cells in the context of defined MHC products on the surface of autologous APC. We present the first evidence that human insulin can be presented and recognized by T cells in the context of HLA-DRB1*0406 products, which act as the dominant restriction elements for human insulin. Furthermore, we discuss the involvement of DRB1*0406 gene products in the recognition of human insulin by T cells in IAS patients and its direct contribution to the pathogenesis of IAS.

Materials and Methods

Patients and healthy donors

Eleven patients suffering from clinically definite IAS and 27 healthy donors participated in this study. All the individuals with HLA-DRB1*0406 or HLA-DRB1*0405 were typed for HLA class II alleles using polymerase chain reaction-sequence specific oligonucleotide probing method (6).

L cell transfectants

Construction of the L cell transfectants expressing the KT2 (DRB1*0406) molecules has been described elsewhere (7, 8). Nontransfected L cells were also used as control APC. DRB1*0406-transfected L cells and nontransfected L cells were maintained in DMEM (GIBCO, Grand Island, NY), supplemented with HAT (GIBCO) and 10% FCS. L cell transfectants expressing the Dw15 (DRB1*0405) molecules were kindly provided by Dr. Jack Silver (Cornell University Medical College, New York). DRB1*0405-transfected L cells were maintained in DMEM medium without HAT supplement.

Cell cultures

Autologous MLR was performed as follows: 5×10^4 irradiated PBMC (20 Gy) used as APC were incubated with or without 40 μM human insulin in medium for 18 h at 37°C and mixed with autologous-enriched T cells (5×10^4) from the same donor. From PBMC nonadherent cells were used as enriched T cells. PBMC were incubated in tissue culture dishes (Corning) for 30 min at 37°C and nonadherent cells were collected. In some experiments, L cell transfectants were used as APC. The irradiated (80 Gy) L cell transfectants (5×10^3) were incubated with or without 40 μM human insulin in medium containing DTT (final concentration, 2 mM, Wako Pure Chemical Industries, Tokyo) for 18 h at 37°C then washed and mixed with 5×10^4 enriched T cells from the same donor. Fixed L cell transfectants were also prepared for APC. L cells were fixed by 0.01% paraform-aldehyde solution for 20 min and washed twice. The mixed cells were incubated in a 5% CO_2 humidified air incubator for 6 days at 37°C. Each well was pulsed with 1 μ Ci of [^{3}H]thymidine during the final 18 h of culture. The results represent the mean cpm of triplicate cultures. Blocking experiments of autologous MLR by mAb were performed in the presence of human insulin (40 μM, Novo Nordisk A/S, Denmark) and 0.2 μg/ml of anti-HLA-A, B, C (W6/32, Sera-lab, Sussex, UK), anti-HLA-DR (L243), or anti-HLA-DQ (SK 10) mAb. Mouse IgG1 (X 40) and IgG2a (X 39, Becton Dickinson, Mountain View, CA) controls were also added in the same concentration.

Establishment of T cell clones

Five human insulin-specific T cell clones (T 108, H120, H122, H141, and H142) were established from blast cells in autologous MLR in the presence of 40 μM human insulin by limiting dilution technique using PBMC from two healthy donors with DRB1*0406. HLA phenotypes of the donor for the clone T 108 is HLA-A 11/33, B 44/62, Cw 4, DR 4/13, DQ 1/3, DRB1*0406/*1302, DQA1*0102/*0301, and DQB1*0302/*0604. HLA phenotype of the donor for the clones H120, H122, H141, H142 is HLA-A2/11, B 62, Cw 4, DR 4, DQ 3, DRB1*0406, DQA1*0301, and DQB1*0302 (homozygote).

Briefly, 2×10^6 PBMC were cultured in the presence of 40 μM human insulin for 7 days. Blast cells were collected then washed and seeded in a microcloning plate at 1/well in the presence of 5×10^4 irradiated (20 Gy) autologous PBMC, 40 μM human insulin, and 1000 μ/ml of rIL-2 (Shionogi Pharmaceutical Tokyo). The cell cultures were supplemented with rIL-2 every 2 to 3 days and with irradiated autologous PBMC in the presence of human insulin twice a month. AIM-V medium (GIBCO) supplemented with pooled normal human AB serum was used throughout the culture.

Determination of the specificity of T cell clones

To determine the specificity of T cell clones, 1.25×10^4 cloned cells were cultured with 1.25×10^3 irradiated (80 Gy) DRB1*0406-transfected L cells with or without 40 μM human insulin in the presence of 2 mM DTT for 2 days. During the final 18 h of incubation, each well was pulsed with 1 μCi of [^{3}H]thymidine. Four T cell clones were also cultured with nontransfected L cells to confirm the function of DRB1*0406 gene products.

Phenotypic characterization of the clones

These T cell clones were examined for the surface expression of CD4, CD8, TCR-$\alpha\beta$, and TCR-$\gamma\delta$ by flow cytometry (Ortho Spectrum III, Ortho Diagnostic Systems) using FITC-conjugated Leu 3, Leu 2a, WT 31, and 11 F2, respectively.

Table I
*Proliferative response of enriched T cells to human insulin in the context of HLA-DRBI*0406 products*[a]

		[³H]thymidine Incorporation (cpm)				
	Allele DRBI*	R Alone	R+APC	R+APC+HI	SI[b]	Response
Patients						
FL	0406	678	1723	40,851	23.7	+
HI	0406	925	1677	16,709	9.9	+
SG	0406	1257	1997	19,595	9.8	+
YT	0406	1221	2317	19,024	8.2	+
IS	0406	1205	2141	16,533	7.7	+
KU	0406	696	1666	12,002	7.2	+
SM	0406	481	873	5873	6.7	+
KH	0406	1355	2073	13,355	6.4	+
TS	0406	1419	1890	9198	4.8	+
UT	0406	1726	2276	7738	3.4	+
MU	0406	5587	6828	20,926	3.1	+
Healthy donors						
SH	0406	1166	1275	47,021	42.1	+
MI	0406	1221	2001	43,233	32.3	+
HO	0406	811	1786	23,153	12.9	+
SO	0406	732	1596	15,031	9.4	+
HI	0406	1213	3199	27,847	8.7	+
JJ	0406	1859	3973	30,333	7.6	+
KA	0406	2156	1610	7702	7.1	+
SA	0406	2709	4822	25,150	5.2	+
AK	0406	1531	1553	6328	3.8	+
HA	0406	1523	3226	10,428	3.2	+
NA	0405	983	1668	1266	0.7	–
IN	0405	1288	2083	1696	0.8	–
ME	0405	351	228	189	0.8	–
SJ	0405	619	1354	1854	1.3	–
KN	0405	1114	1691	2429	1.4	–

[a] The 5×10^4 irradiated PBMC as APC were incubated with or without 40 μM human insulin in medium for 18 h at 37°C and cultured for 6 days with 5×10^4 autologous-enriched T cells of the same donor as responder (R). Results represent the mean triplicate (SD < 15% of the mean). [³H]thymidine incorporation of APC alone, APCs + HI (human insulin), and R+HI were 554 ± 103, 886 ± 130, and 1608 ± 210, respectively.
[b] SI >3 was considered as a positive response (+).

Results

Human insulin is presented by autologous APC with DRB1*0406

All of the IAS patients were found to possess DRB1*0406, one of the major subtypes of HLA-DR4 in the Japanese population (5, 6). Another major subtype of HLA-DR4 is DRB1*0405, associated with insulin-dependent diabetes mellitus (9) and rheumatoid arthritis (10). We first attempted to determine whether the proliferative response of T cells to human insulin is related to DRB1*0406 haplotypes. In the presence of human insulin, autologous MLR were performed with 25 blood samples from 11 IAS patients with DRB1*0406, 10 healthy donors with DRB1*0406, and 5 healthy donors with DRB1*0405 (used as controls). Once APC were exposed to human insulin (40 μM), [³H]thymidine incorporation of T cells increased 3- to 40-fold 6 days after the exposure of insulin to autologous APC with DRB1*0406, but not to autologous APC with DRB1*0405 (Table I). There was no difference in the increasing fold among the 11 IAS patients and the 10 healthy donors, all DRB1*0406. Furthermore, we analyzed the proliferative response of T cells to human insulin in autologous MLR from 12 donors with HLA-DR phenotypes other than DR4 (Table II). There was no effect on the proliferative response of T cells after the exposure of human insulin to autologous APC with non-HLA-DR 4 phenotypes. In these experiments, the exposure to 40 μM of human insulin had no influence on the proliferative response of APC or T cells alone, showing that human insulin does not contribute to T cell proliferation as a growth factor (Tables I and II). These

Table II
No proliferative response of T cells to human insulin in the presence of autologous APC with HLA-DR4-negative phenotypes[a]

Healthy Donors	HLA-DR	R Alone	R+APC	R+APC+HI	SI
NI	DR-2	1562	3921	3529	0.9
TO	DR-8,9	848	1386	1956	1.4
MA	DR-1,15	984	1567	1713	1.0
SA	DR-2,8	1328	2192	2545	1.6
TM	DR-9	1083	1455	1525	1.0
KI	DR-7,8	785	2492	2325	0.9
TA	DR-8	1056	3857	4327	1.1
MT	DR-8,12	2540	6095	10,887	1.8
RI	DR-2,12	931	2994	3424	1.1
HM	DR-13,15	1227	1669	2602	1.5
KM	DR-14	1093	1409	1827	1.2
UI	DR-8,15	484	2533	2300	0.9

[a] Autologous MLR was performed as described in *Materials and Methods*. Results represent the mean of triplicate (SD <15% of the mean). [³H]thymidine incorporation of APC alone, APC+HI (human insulin), and R (responder) + HI were 495 ± 55, 580 ± 75, and 1276 ± 191, respectively.

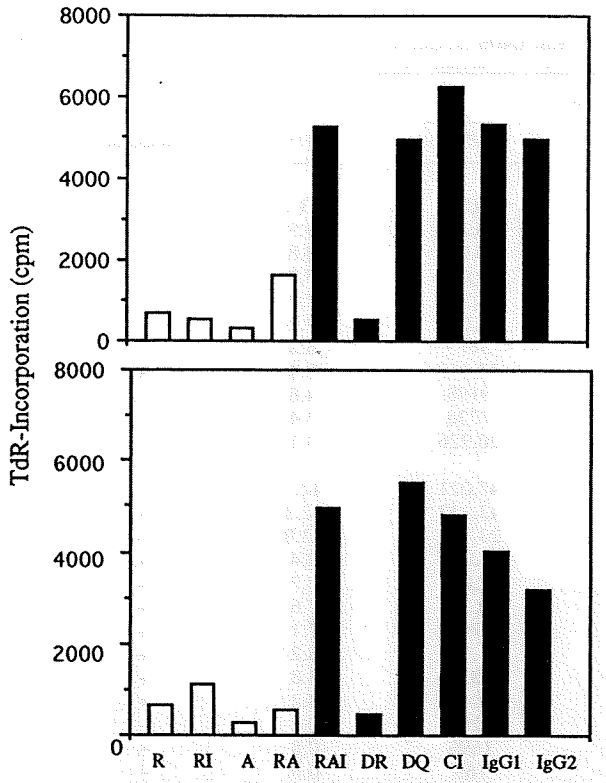

FIGURE 1. T cell responses (top from donor MI and bottom from donor SO) to human insulin blocked by anti-HLA DR mAb. Results represent the mean of triplicate (SD < 15% of the mean). R, enriched T cells; RI, R + human insulin; A, APC, DR, R+A+I+anti-HLA-DR mAb; DQ, +anti-HLA-DQ mAb; CI, +anti-HLA class I mAb; IgG1, +mouse IgG1 control; IgG2a, +mouse IgG2a control.

results indicate that human insulin is presented by autologous APC with DRB1*0406 and/or DQA1*0301 and/or DQB1*0302 products.

DR gene products are involved in the recognition of human insulin by T cells

Because there is a strong linkage disequilibrium between DRB1*0406, DQA1*0301, and DQB1*0302 in the Japanese population (6), it is difficult to determine the primary gene products of the responsiveness to human insulin from the association analysis. To determine which gene products were involved in the recognition of human insulin by T cells, the proliferative response of T cells from two donors (MI and SO) to human insulin was assessed in the presence of purified monomorphic anti-HLA class I or anti-HLA class II mAb. Complete inhibition was achieved in the presence of 0.2 μg/ml anti-HLA-DR mAb, whereas no inhibition was observed with anti-HLA-A, B, C, anti-HLA-DQ mAb, and IgG1 and IgG2a controls (Fig. 1), suggesting that DR gene products, rather than DQ gene products, were involved in the recognition of human insulin by T cells.

HLA-DRB1*0406 gene products act as the dominant restriction element for human insulin presentation to T cells

L cell transfectants expressing high levels of HLA class II gene products can efficiently present either peptidic or whole protein Ag to human T cells (11). In an attempt to confirm that DRB1*0406 gene products are involved in the recognition of human insulin by T cells, we used DRB1*0406-transfected L cells as APC. Before the experiments, DRB1*0406-transfected L cells were labeled with FITC-conjugated anti-DR mAb (L 243) and analyzed by FACScan flow cytometer and the expression of HLA-DR was observed (data not shown). Moreover, to confirm the function of HLA-DR molecules, T cells from a non-DRB1*0406 donor were cultured with irradiated DRB1*0406 transfectants. Significant allogenic responses were observed (data not shown). As shown in Table III, DRB1*0406-transfected L cells were able to present human insulin to T cells from healthy donors with DRB1*0406 or IAS patients with DRB1*0406 in the presence of 2 mM DTT. T cells derived from two healthy donors with DRB1*0405 were also cultured with DRB1*0405-transfected L cells in the same conditions but proliferative responses were not observed. Like other protein Ag, insulin requires processing in metabolically active APC before it can be recognized by class II-restricted T cells (12). This processing requirement is bypassed by the incubation of insulin with DTT in the presence of aldehyde-fixed APC, which cannot process protein Ag (12). To investigate the effect of DTT, fixed L cells were used as APC. In this case, DTT might act through reduction of one or more disulfide bonds in insulin, allowing reduced derivatives to interact directly with cell surface HLA class II molecules.

When fixed L cells were incubated with DTT and human insulin, [^{3}H]thymidine incorporation compared with the condition without DTT (Table IV). Thus, DRB1*0406-transfected L cells were also able to present insulin to T cells in the presence of DTT, supporting that DRB1*0406 gene products are involved in the recognition of human insulin by T cells. These results in the blocking experiment with mAb and the experiment using DRB1*0406-transfected L cells described above strongly suggest that human insulin can be presented and recognized in the context of DRB1*0406 products, which act as the dominant restriction element for human insulin.

Establishment of T cell clones reactive to human insulin

Five human insulin-specific T cell clones were established by limiting dilution of blast cells in autologous MLR of PBMC from two healthy donors with DRB1*0406 in the presence of human insulin. These T cell clones were char-

Table III
*HLA-DRB1*0406-transfected L cells can present human insulin to enriched T cells from a healthy donor or an IAS patient with-DRB1*0406 in the presence of DTT*[a]

		[^{3}H]thymidine Incorporation (cpm)				
	DRBI*	R Only	R+L	R+L+DTT	R+L+HI	R+L+HI+DTT
IAS patients						
IS	0406	1834 ± 23	905 ± 79	1554 ± 138	16,267 ± 306	12,692 ± 374
SM	0406	2396 ± 199	5167 ± 263	6104 ± 413	ND	15,218 ± 822
Healthy donors						
JJ	0406	247 ± 141	3711 ± 41	3493 ± 572	7280 ± 1,874	11,854 ± 538
KA	0406	208 ± 25	1618 ± 465	1518 ± 391	5727 ± 607	7492 ± 248
NA	0405	328 ± 25	457 ± 4	ND	506 ± 46	ND
ME	0405	1563 ± 416	2819 ± 251	1309 ± 15	572 ± 32	630 ± 59

[a] The 5 × 10^3 irradiated (80 Gy) L cell transfectants (L) were incubated with or without 40 μM human insulin (HI) in medium containing 2 mM DTT and cultured for 6 days with 5 × 10^4 enriched T cells (R, responder cells) [^{3}H]thymidine incorporation of L cells alone were 3300 ± 97 for DRB1*0406 transfectant and 1586 ± 94 for DRB1*0405 transfectant, respectively.

Table IV
Fixed L cells effectively presented human insulin to responder cells in the presence of DTT[a]

		[^{3}H]thymidine Incorporation (cpm)			
Healthy Donors	L cells	R+L	R+L+DTT	R+L+HI	R+L+DTT+HI
JJ	L DR	3711 ± 41	3493 ± 572	7280 ± 1874	11,854 ± 538
	L fixed	2395 ± 366	2205 ± 1079	3703 ± 249	6004 ± 264
YA	L DR	2953 ± 345	1670 ± 203	10,946 ± 697	11,386 ± 388
	L fixed	2618 ± 746	2900 ± 839	5097 ± 1325	10,769 ± 1850

[a] The 5 × 10^3 irradiated (80 Gy) L cell transfectants (L DR) and paraformaldehyde-treated fixed L cell transfectants (L fixed) were incubated with or without 40 mM human insulin (HI) in medium containing 2 mM DTT and cultured for 6 days with 5 × 10^4 enriched T cells (R, responder cells). [^{3}H]thymidine incorporation of R alone were 247 ± 141 (JJ) and 236 ± 1 (YA) and L cells alone were 3300 ± 97 (L DR) and 1325 ± 11 (L fixed), respectively.

acterized to be TCR-$\alpha\beta$ and CD4-positive T cells (data not shown). All of the T cell clones were able to proliferate in response to human insulin in the presence of primary APC with DRB1*0406.

On the surface of conventional APC, different HLA class II gene products are codominantly expressed. To circumvent this problem, and to address which gene products of a given HLA class II haplotype on APC may be involved in the presentation of human insulin, we used DRB1*0406-transfected L cells for determining the specificity of these T cell clones. The irradiated (80 Gy) L cell transfectants (5 × 10^3) were incubated with or without 40 mM human insulin in medium containing 2 mM DTT and cultured for 2 days with 5 × 10^4 T cell clones. As shown in Table V, all the T cell clones were able to proliferate in response to human insulin in the presence of DRB1*0406-transfected L cells with DTT, but did not show the proliferative responses in the presence of nontransfected L cells. This result confirmed that DRB1*0406 gene products are involved in the recognition of human insulin by T cells.

Discussion

IAS, which was first reported by Hirata et al. in 1970 (13), is the third cause of spontaneous hypoglycemia in Japan (14). It is diagnosed by the following criteria: spontaneous hypoglycemia without evidence of exogenous insulin administration, a large amount of total serum immunoreactive insulin, and the presence of a high titer of anti-insulin antibody.

As in other human diseases with a putative autoimmune pathogenesis, susceptibility to IAS is associated with certain HLA haplotypes. In the Japanese population, HLA class II alleles, HLA-DRB1*0406, DQA1*0301, and DQB1*0302, are associated with an enhanced disease susceptibility (6). Although the existence of other HLA class II associated "disease genes" cannot be excluded, it is most probable that the HLA class II genes, which control the T cell reactivity to insulin and subsequently the production of anti-insulin antibody by B cells, are the key factors involved in this association.

We recently showed that T cells from IAS patients were able to proliferate in response to human insulin in the presence of APC with DRB1*0406, DQA1*0301, and DQB1*0302 (5). In this study, we demonstrated that the T cells from not only IAS patients but also healthy donors were able to proliferate after exposure of human insulin to autologous APC with DRB1*0406, DQA1*0301, and DQB1*0302 (Tables I and II). The grade of T cell response to human insulin did not differ IAS patients and healthy donors. These results provide evidence that human insulin is presented by autologous APC with DRB1*0406. Among IAS patients, SI was not correlated with the titers of anti-insulin antibody. And in the repeated studies, the grade of T cell response differed for

Table V
*Proliferative responses of T cell clones to human insulin in the presence of HLA-DRB1*0406-transfected L cells*[a]

		[³H]thymidine Incorporation (cpm)			
C	L Cells	C+L	C+L+DTT	C+L+HI	C+L+HI+DTT
T108	L DR	8329 ± 458	9308 ± 765	25,294 ± 1066	19,697 ± 2210
H120	L DR	1008 ± 96	1070 ± 11	12,039 ± 548	11,682 ± 537
	L non	3021 ± 115	2896 ± 161	5089 ± 297	5572 ± 357
H122	L DR	3160 ± 726	3435 ± 251	7005 ± 356	10,200 ± 596
	L non	3537 ± 94	2141 ± 285	6378 ± 270	5400 ± 16
H141	L DR	5142 ± 279	4529 ± 333	13,271 ± 270	17,928 ± 443
	L non	1338 ± 50	1263 ± 41	3379 ± 157	3008 ± 345
H142	L DR	2751 ± 371	3406 ± 328	7414 ± 600	10,988 ± 438
	L non	6377 ± 222	4839 ± 339	6238 ± 39	7239 ± 176

[a] The 5×10^3 irradiated (80 Gy) DRB1*0406 transfected L cells (L DR) and nontransfected L cells (L non) were incubated with or without 40 μM human insulin (HI) in medium containing 2 mM DTT and cultured for 2 days with 5×10^4 T cell clones (C). [³H]thymidine incorporation of L DR alone and L none alone were 5003 ± 1066 and 4190 ± 232, respectively. And clone cells alone were T108, 171 ± 9; H120, 1045 ± 5; H122, 3142 ± 8, H141, 242 ± 41; H142, 172 ± 42, respectively.

each donor but always showed significant proliferation in response to human insulin (SI > 3). However, it is difficult to determine which gene product is primarily involved in the responsiveness to human insulin from the result described above, due to the strong linkage disequilibrium between DRB1*0406, DQA1*0301, and DQB1*0302 in the Japanese population.

Therefore, we performed the blocking experiments with mAb and the experiments using DRB1*0406-transfected L cells to determine which gene product is the primary one. We tested the proliferative response of T cells from two healthy donors with DRB1*0406, DQA1*0301, and DQB1*0302 to human insulin in the presence of anti-HLA class I or class II mAb and found that it was inhibited in the presence of anti-HLA-DR mAb but not anti-HLA-DQ mAb (Fig. 1). Also, anti-class I mAb and IgG1 and IgG2a controls did not affect the T cell proliferation.

In the experiment with L cell transfectants, these cells were able to present human insulin to T cells in the presence of DTT, which is thought to reduce one or more disulfide bonds in insulin. Jensen et al. (12), using conventional APC, showed that the reduction of disulfide bonds in insulin was both necessary and sufficient to allow the formation of functional insulin/class II complexes.

Furthermore, we established human insulin-specific CD4-positive T cell clones from two healthy donors with DRB1*0406. Originally, these T cell clones were isolated by repeated stimulation with PBMC as APC with DRB1*0406 in the presence of human insulin. To confirm that DRB1*0406 products act as the dominant restriction element for human insulin, we used DRB1*0406-transfected L cells for determining the specificity of these T cell clones. All of the T cell clones were able to proliferate in response to human insulin in the presence of DRB1*0406-transfected L cells (Table V). In this case, DTT was also used for reducing and cleaving the disulfide bonds of insulin. The result confirmed that DRB1*0406 gene products are involved in the recognition of human insulin by T cells.

Throughout the results of Tables III to V, in some experiments we observed that DRB1*0406-transfected L cells could present human insulin to T cells, despite the fact that medium did not contain DTT. Because paraformaldehyde fixation induced the inhibition of Ag processing, fixed L cells were not able to present human insulin to T cells without DTT (Table IV). We then consider that L cells have the ability to process human insulin to a certain extent. Table IV shows the effect of DTT on human insulin producing some cleaved peptide fragments that bind directly to DRB1 *0406 gene products. The effect is considered to assist in the Ag presentation to T cells. Focusing on the etiology of IAS before the onset of the disease many patients were treated with reducing agents.[3] These agents like DTT may be related to the onset of IAS by presenting human insulin effectively to T cells in vivo.

Considering that HLA-DRB1*0406 products act as the dominant restriction element in the presentation of human insulin to T cells as described herein, and that all the IAS patients had DRB1 *0406, which allowed T cell proliferation (5), it strongly suggests that the class II gene DRB1*0406 involves a genetic predisposition to the development of IAS. T cells recognize Ag as peptides bound to MHC-encoded molecules on the surface of APC (15). Two classes of MHC molecules are involved in the Ag presentation to T cells. Two separate pathways of Ag processing and presentation have been proposed, leading to selective association of peptides from endogeneous Ag to class I molecules and of peptides from exogenous protein Ag to class II MHC molecules (16). Regarding the above mentioned, it is assumed that exogeneous autoantigenic hu-

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man insulin might be presented by human class II molecules that are the gene products of DRB1*0406 and induced the proliferation of Th cells followed by the production of a high titer of anti-insulin antibody by B cells in IAS.

In addition, our study showed that the proliferative response of T cells to human insulin is restricted to DRB1*0406, but not to DRB1*0405 products (Tables I and III). DRB1*0406 molecules differ from DRB1*0405 molecules in four amino acid sequences, at positions 37, 57, 74, and 86. The limited structural differences between DRB1*0406 and DRB1*0405 based on these amino acid differences showed a dramatic influence on the immune recognition site (17). The third hypervariable region of the class II β-chain represents a potential recognition site for T cells (18). Consequently, among the four kinds of amino acid differences between DRB1*0406 and DRB1 *0405, the change at position 74 resulting in a charge difference from an acidic amino acid (glutamic acid) to a neutral amino acid (alanine) in the third hypervariable region may have a particular functional importance.

In conclusion, we have demonstrated that human insulin can be presented and recognized by T cells in the context of HLA-DRB1*0406 products, which act as the dominant restriction element, and that the involvement of DRB1*0406 products in the recognition of human insulin provides a new approach for understanding T cell recognition in the context of HLA-DR molecules and T cell-mediated immune responses in autoimmune diseases.

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Ⅳ. その他

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自発性低血糖症の実態把握のための全国調査

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低血糖症の分類

●内因性低血糖症
　★自発性低血糖症（空腹時低血糖症）
　★反応性低血糖症（食後低血糖症）
●外因性低血糖症

誘発性低血糖症

自発性低血糖症は、
糖尿病治療薬服用やインスリン治療と関連なく、多くは明らかな原因なく、突然に震えから意識障害までいろいろな低血糖症状をきたす低血糖症である。

代表的な原因疾患として、インスリノーマ、膵外腫瘍による低血糖症、インスリン自己免疫症候群がある。しかし、原因究明が困難な本症も多い。

背　景

自発性低血糖症の原因疾患に関する全国調査は、第1回は1982年に、第2回は1988年に、当施設が全国200床以上の2000余りの医療機関を対象に独自に実施した歴史がある。

1回、2回の調査の結果は同じで、上位3疾患は、

1. インスリノーマ　5
2. 膵外腫瘍による低血糖症（NICTH）　3
3. インスリン自己免疫症候群（IAS）　2

であった。

これまでの調査の結果

1982年
第1回調査（350件）の順位

1.	インスリノーマ	35.7%
2.	NICTH	26.3
3.	IAS	11.7
4.	アルコール性	6.6
5.	インスリン拮抗ホルモン低下症	6.6
6.	敗血症	1.1
7.	慢性腎不全	1.1

最近の状況

ところが、最近、学会発表や学会雑誌において、インスリノーマや腫瘍などに拠らない自発性低血糖症症例の報告が目立つようになった。

症例の多くは、発症時にサプリメントや漢方薬などを摂取している。

目　的

本研究の目的は、第2回調査から20年を経た平成22年度に、あらためて原因の明らかでない低血糖症の実態を調査し、時代による原因疾患の相違、関連する薬物やサプリメントの抽出、そして警鐘、鑑別診断ガイドライン、治療指針を策定する。

対象と方法

1. 「病院便覧」から200床以上の病院（内科部長あて）および大学病院（内科および小児科教授あて）に、全2183通の調査依頼状を送付。

2. 調査協力できると返答のあった施設に、過去３年間の、以下の自発性低血糖症患者登録を依頼

登録基準
- 低血糖症状があること
- 70mg/dl以下の血糖値が判明している
- 糖質摂取で症状が消失する
- 明らかな糖尿病治療薬やインスリン注射による低血糖症は除外

3. 登録された症例が自発性低血糖症の定義に合致するかどうかの判定は、主任および分担研究者による判定会議にて行う。

結　果

1. 倫理委員会承認後の2009年8月末から2010年1月末までに、207例が登録された(いまなお、登録されている)。

2. 頻度順は、以下のようだった。

1. インスリノーマ	26.0% (54/207)
2. インスリン自己免疫症候群(IAS)	18.6% (38/207)
3. インスリン拮抗ホルモン低下症	9.6% (20/207)
4. 反応性低血糖症	8.7% (18/207)
5. アルコール性低血糖症	4.3% (9/207)
6. 膵外腫瘍による低血糖症(NICTH)	3.8% (8/207)

第1回(1982年)との比較

第1回調査(350件)	今回調査(189件')
1. インスリノーマ	1. インスリノーマ
2. NICTH	2. IAS
3. IAS	3. インスリン拮抗ホルモン低下症
4. アルコール性	4. アルコール性
5. インスリン拮抗ホルモン低下症	5. NICTH
6. 敗血症	
7. 慢性腎不全	

＊4例以上の疾患名を挙げた。
＋ 第1回調査と合わせるために、反応性低血糖症は除いた。

1および2回調査との比較

	第1回調査(1982)	第2回調査(1988)	今回調査(2009)
依頼先数	2094	2094	2183
登録件数	350	264	207
	258	185	100
インスリノーマ	125(48. 4%)	93(50. 3%)	54(54. 0%)
NICTH	92(35. 6%)	56(30. 2%)	8 (0. 8%)
IAS	41(15. 9%)	36(19. 5%)	38(38. 0%)
	5	5	5
	3	3	1
	2	2	4

結　果

3. 189例(反応性を除外)のうち、低血糖症と関連していると報告された薬物およびサプリメントで頻度の多いもの

α-リポ酸＊	17 (8.9%)
アルコール	9 (4.7%)
コエンザイムQ	2 (1.0%)
コハク酸シベゾリン(不整脈薬)	3 (1.6%)
男根増進薬	2 (1.0%)
ブシラミン(リマチル®) (RA) ＊	1 (0.5%)
メチマゾール(バセドウ病)＊	1 (0.5%)

その他、ベシル酸アムロジピン、バルサルタン、カルベジロールなど

＊ IASと関連するもの