HLA-DR Binding Analysis of Peptides From Islet Antigens in IDDM

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HLA molecules are essential for thymic education and HLA restriction of T-cell responses. We therefore analyzed the HLA-DR binding affinities of synthetic peptides covering the entire sequences of GAD65, islet cell antigen 69 (ICA69), and (pro)insulin, which are candidate antigens in the autoimmune process of T-cell–mediated destruction of the pancreatic β-cells. Subsequently, peptide HLA-DR binding was correlated to peptide antigenicity by comparing known T-cell epitopes with their HLA-binding affinities defined in this study. The results demonstrate the following. *1*) (Pro)insulin peptides display a strong binding affinity for HLA-DR2, which is associated with negative genetic predisposition to IDDM, whereas poor binding was observed for HLA-DR molecules neutrally or positively associated with IDDM. This suggests that the absence of insulin-reactive T-cells in DR2+ individuals may be explained by negative selection on high-affinity DR2 binding insulin peptides. *2*) Most autoantigenic peptides display promiscuous HLA-DR binding patterns. This promiscuity in itself is not sufficient to explain the genetic association of HLA-DR with development of IDDM. *3*) HLA-DR3 binding of autoantigenic GAD65 peptides is relatively weak compared with that of other known T-cell epitopes. *4*) All peptide epitopes recognized by HLA-DR–restricted T-cells from either IDDM patients or GAD65-immunized HLA-DR transgenic mice bind with high affinity to their HLA-DR restriction molecule ($P < 0.0006$). In contrast, T-cell epitopes recognized by nondiabetic controls bind DR molecules with weak or undetectable affinity. These results thus indicate a strong correlation between antigenicity and HLA-DR binding affinity of GAD65 peptides in IDDM. Furthermore, negative thymic selection of insulin peptides in low-risk (HLA-DR2 expressing) subjects may explain the lack of autoreactivity to insulin in such individuals. *Diabetes* 47:1594–1601, 1998

IDDM is considered to result from a genetically controlled T-cell–mediated autoimmune destruction of the insulin-producing pancreatic β-cells (1). Several lines of evidence indicate a central role for T-cells in the pathogenesis of IDDM (2–5). In humans, autoreactive T-cells responding to GAD65 (65-kDa glutamic acid decarboxylase), ICA69 (69-kDa islet cell antigen), insulin, and other β-cell antigens have been described, but their role in β-cell destruction is yet unclear (6–11). The HLA region, in particular the class II region, is strongly involved in the genetic predisposition to develop IDDM (12–14). Because the HLA gene products play an essential role in thymic T-cell repertoire selection and peripheral presentation of antigenic peptide epitopes to T-cells, it is conceivable that the association of HLA class II with IDDM or IDDM progression could be through binding and presentation of autoantigenic peptides derived from β-cell proteins to autoreactive T-cells. Thus, a crucial feature of T-cell autoreactivity lies in the interaction of candidate autoantigenic peptides with HLA class II molecules.

T-cells reactive with β-cell antigens have been shown to be primarily restricted by HLA-DR, rather than by HLA-DQ or -DP. The binding affinity, however, of peptides derived from β-cell proteins for HLA class II molecules has not yet been analyzed in great detail (15,16). Previously, a computer program was developed that allows prediction of potential HLA class I binding motifs in proteins (17). This program was shown to be useful in defining peptides suitable to stimulate HLA-class I–restricted T-cells (18). In this study, we have adjusted the program to predict HLA-DR peptide binding motifs, as well.

In the case of autoimmunity, it is not yet resolved whether autoantigenic peptides contain strong major histocompatibility complex (MHC) binding sequences or, alternatively, harbor motifs associated with intermediate or low MHC binding affinity. The latter could explain the ineffective elimination of autoreactive T-cells in the thymus that may cause autoimmunity or even autoimmune disease.

In this report, we describe the binding affinity of a complete series of overlapping synthetic peptides from three candidate human islet autoantigens in IDDM: GAD65, ICA69, and (pro)insulin. Binding was analyzed to four different HLA molecules associated with either protection against (HLA-DR2; DRB1*1501) or susceptibility to (HLA-DR3; DRB1*0301 and –DR4; DRB1*0401) IDDM, or with no evident genetic association (HLA-DR1; DRB1*0101). The binding affinity was subsequently evaluated in relation to the computer-assisted predicted binding for HLA-DR. Finally, we related MHC bind-

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HA, hemagglutinin; IC_{50}, concentration of the test peptide at which binding of the labeled standard peptide reaches its half-maximal value; ICA, islet cell antigen; MHC, major histocompatibility complex.

TABLE 1
HLA-DR binding affinities of GAD65-derived 20-mer peptides (IC_{50} in μmol/l)

	DR1	DR2	DR3	DR4
1–20 MASPGSGFWSFGSEDGSGDS	5	5	>100	5.4
11–30 FGSEDGSGDSENPGRARAWC	>100	>100	>100	62
21–40 ENPGRARAWCQVAQKFTGGI	30	3	>100	>100
31–50 QVAQKFTGGIGNKLCALLYG	0.2	15	>100	>100
41–60 GNKLCALLYGDAEKPAESGG	34	5	92	>100
51–70 DAEKPAESGGSQPPRAAARK	>100	>100	>100	>100
61–80 SQPPRAAARKAACACDQKPC	>100	30	>100	>100
71–90 AACACDQKPCSCSKVDVNYA	46	37	60	2.4
81–100 SCSKVDVNYAFLHATDLLPA	0.03	0.2	>100	0.5
91–110 FLHATDLLPACDGERPTLAF	70	19	>100	0.2
101–120 CDGERPTLAFLQDVMNILLQ	>100	>100	>100	>100
111–130 LQDVMNILLQYVVKSFDRST	4	10	30	0.7
121–140 YVVKSFDRSTKVIDFHYPNE	6	5	30	19
131–150 KVIDFHYPNELLQEYNWELA	3	0.7	>100	5
141–160 LLQEYNWELADQPQNLEEIL	41	1.2	>100	5
151–170 DQPQNLEEILMHCQTTLKYA	>100	1.2	>100	>100
161–180 MHCQTTLKYAIKTGHPRYFN	15	0.1	30	>100
171–190 IKTGHPRYFNQLSTGLDMVG	0.6	0.06	13	0.5
181–200 QLSTGLDMVGLAADWLTSTA	2	2	>100	1.4
191–210 LAADWLTSTANTNMFTYEIA	18	5	70	41
201–220 NTNMFTYEIAPVFVLLEYVT	3	1.2	70	85
221–240 LKKMREIIGWPGGSGDGIFS	3	43	>100	48
231–250 PGGSGDGIFSPGGAISNMYA	3	8	>100	80
241–260 PGGAISNMYAMMIARFKMFP	2	0.1	>100	>100
251–270 MMIARFKMFPEVKEKGMAAL	3	2	20	25
261–280 EVKEKGMAALPRLIAFTSEH	6	1	>100	40
271–290 PRLIAFTSEHSHFSLKKGAA	2	0.03	>100	0.2
281–300 SHFSLKKGAAALGIGTDSVI	0.5	2.4	>100	>100
291–310 ALGIGTDSVILIKCDERGKM	96	17	28	24
301–320 LIKCDERGKMIPSDLERRIL	31	3.4	0.2	>100
311–330 IPSDLERRILEAKQKGFVPF	53	0.4	85	38
321–340 EAKQKGFVPFLVSATAGTTV	0.9	5	>100	4
331–350 LVSATAGTTVYGAFDPLLAV	0.9	0.7	70	7
341–360 YGAFDPLLAVADICKKYKIW	5	3	>100	>100
351–370 ADICKKYKIWMHVDAAWGGG	15	8.3	27	2.7
361–380 MHVDAAWGGGLLMSRKHKWK	5	10	33	43
371–390 LLMSRKHKWKLSGVERANSV	4	3	>100	0.8
381–400 LSGVERANSVTWNPHKMMGV	30	>100	>100	13
391–410 TWNPHKMMGVPLQCSALLVR	8	9	>100	34
401–420 PLQCSALLVREEGLMQNCNQ	34	>100	>100	17
411–430 EEGLMQNCNQMHASYLFQQD	12	10	>100	41
421–440 MHASYLFQQDKHYDLSYDTG	>100	0.8	>100	22
431–450 KHYDLSYDTGDKALQCGRHV	>100	>100	>100	31
441–460 DKALQCGRHVDVFKLWLMWR	>100	>100	>100	>100
451–470 DVFKLWLMWRAKGTTGFEAH	17	1.5	>100	33
461–480 AKGTTGFEAHVDKCLELAEY	>100	65	60	>100
471–490 VDKCLELAEYLYNIIKNREG	72	5	>100	34
481–500 LYNIIKNREGYEMVFDGKPQ	8	5.4	35	4
491–510 YEMVFDGKPQHTNVCFWYIP	>100	>100	56	67
501–520 HTNVCFWYIPPSLRTLEDNE	2	0.2	70	0.6
511–530 PSLRTLEDNEERMSRLSKVA	70	6	6	5
521–540 ERMSRLSKVAPVIKARMMEY	70	0.5	>100	>100
531–550 PVIKARMMEYGTTMVSYQPL	8	4	30	10
541–560 GTTMVSYQPLGDKVNFFRMV	25	4	70	10
551–570 GDKVNFFRMVISNPAATHQD	0.007	0.2	35	0.7
561–580 ISNPAATHQDIDFLIEEIER	>100	34	>100	65
566–585 ATHQDIDFLIEEIERLGQDL	>100	1.6	>100	20

ing affinity of autoantigen-derived peptides with antigenicity using peptides defined as epitopes of autoreactive T-cells.

RESEARCH DESIGN AND METHODS

Peptide synthesis. Peptides were made on an ABIMED 422 synthesizer (ABIMED, Langenfeld, Germany) using the simultaneous multiple peptide synthesis method (19). The purity of the peptides was checked on reversed-phase C18 high-performance liquid chromatography (LiChrospher, 60RP-select B 5 µm, 250 × 4 mm, Merck, Darmstadt, Germany) and was shown to be routinely over 75%.

Affinity purification of DR molecules. As a source of DR molecules, EBV-BLCL homozygous for DR were used: LG2.1 (DRB1*0101, DR1), IWB (DRB1*1501, DR2), HAR (DRB1*0301, DR3 [17]), and BSM (DRB1*0401, DR4). Cells were cultured in RPMI 1640 (GIBCO, Paisley, U.K.) supplemented with 2 mmol/l L-glutamine (GIBCO), 100 U · 100 μg^{-1} · ml^{-1} penicillin/streptomycin solution (GIBCO), and 10% heat-inactivated fetal calf serum (GIBCO). Cells were lysed at a concentration of 10^8 cells/ml in 50 mmol/l Tris-HCl, pH 8.5, containing 2% Renex (Accurate Chemicals and Scientific, Westbury, NY), 150 mmol/l NaCl, 5 mmol/l EDTA, and 2 mmol/l phenylmethylsulfonyl fluoride (PMSF). The lysates were cleared of nuclear and other debris by centrifugation at 10,000*g* for 20 min. DR molecules were purified as described previously (20).

DR-peptide binding assay. Purified DR molecules (60–600 nmol/l) were incubated at pH 4.5 for 48 h with 100 fmol (6.7 nmol/l) fluorescent-labeled peptide in 15 µl (final volume) of 100 mmol/l Na-phosphate buffer containing 75 mmol/l NaCl, 1 mmol/l 3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate (CHAPS) (Merck, Darmstadt, Germany) and 15% (vol/vol) CH_3CN in the presence of a protease inhibitor mixture. The final concentrations of protease inhibitors were: 1 µmol/l chymostatin, 5 µmol/l leupeptin, 10 µmol/l pepstatin A, 1 mmol/l EDTA, and 200 µmol/l pefabloc. Standard fluorescent-labeled peptides used, which were labeled as described previously (19), were influenza hemagglutinin (HA) p307–319 (PKYVKQNTLKLAT, DR1 and DR2), hp65 p3–13 (KTIAYDEEARR, DR3), and HA p307–319 Y→F (DR4). IC_{50} values (concentration of test peptides at which binding of the labeled standard peptide reaches its half-maximal value) for unlabeled standard peptides were HA/DR1: 0.01 µmol/l; HA/DR2: 0.02 µmol/l; p3–13/DR3: 0.06 µmol/l; and HA Y→F/DR4: 0.2 µmol/l. In preliminary experiments, each DR preparation was titered in the presence of 100 fmol standard peptide to determine the DR concentration necessary to bind 10–20% of the total fluorescent signal. All subsequent inhibition assays were then performed at this concentration. Peptides of which the DR binding capacity was to be determined were added to DR molecules simultaneously with the standard peptide. The DR-peptide complexes were separated from free peptide by gel filtration on a Synchropak GPC 100 column (250 mm × 4.6 mm; Synchrom, Lafayette, IN). Fluorescent emission was measured at 528 nm on a Jasco FP-920 fluorescence detector (B&L Systems, Zoetermeer, The Netherlands). The percentage of DR-bound, labeled peptide was calculated as the amount of fluorescence bound to MHC divided by total fluorescence. The concentration of peptide inhibitor yielding 50% inhibition was deduced from the dose-response curve. Each peptide was tested at least three times in different experiments to validate the results.

HLA-peptide binding motifs. Motif scores were assigned to each peptide in GAD65, ICA69, and (pro)insulin using the MOTIFS software (17) described previously, in which we incorporated for this study the HLA-DR peptide binding motifs for DR1 (21), DR2 (22), DR3 (20), and DR4 (23). Positive scores were given for each potential anchor residue found in the peptide, and negative scores were given to inhibitory residues. The overall peptide score was the sum of the scores for individual anchor and inhibitor residues. Scores ranged from –10 to 40. Arbritarily, all peptides with scores of ≥20 were considered positive.

RESULTS

GAD65. To analyze HLA-DR binding of GAD65 peptides, we used 58 20-mer peptides, overlapping 10 amino acids with adjacent peptides, covering the complete sequence of human GAD65. More high-affinity binding GAD65 peptides were found in cases of HLA-DR1 and DR2 than in cases of the susceptibility-associated DR3 and DR4 molecules (high-affinity binding in 47 and 67 vs. 3 and 31%, respectively; Table 1). In particular, the majority of GAD65 peptides bound poorly or not at all to DR3. Allele-specific binding was rare, because most peptides bound, although with variable affinities, to three or all four HLA-DR molecules studied (37/58 peptides, Table 1).

Combining peptide–HLA-DR binding affinity with antigenicity (Fig. 1, Tables 1 and 2) revealed a striking concordance between high HLA-DR4 binding affinity of GAD65 peptides and T-cell recognition in DR4+ IDDM patients (24) ($P < 0.0006$), as well as in HLA-DR4 transgenic mice immunized with human GAD65 (27,28). IDDM patient–derived DR2- and DR1-restricted GAD65-specific T-cell epitopes p206–225 (24) and p88–99 (25), respectively, each bound well to their restriction elements (Table 2 and Fig. 1). Also, Lohman et al. (26) reported on GAD65-specific T-cells with poorly defined restrictions and epitope sequences. A putative DR4-restricted T-cell epitope, p511–530, binds well to DR4; and the DR1-restricted p501–520 bound with high affinity to DR1. In contrast, peptide region 161–175, recognized by nondiabetic controls (26), bound with intermediate or undetectable affinity to DR1 and DR4, respectively (Table 2). Similarly, low-affinity binding to HLA-DR3 was detected for DR3-restricted GAD65 epitopes recognized by T-cells of a nondiabetic stiff-man syndrome patient with high titers of GAD65-specific autoantibodies (N.C.S., G. Duinkerken, R.R.P. De Vries, T. Dyrberg, A. Chaudhuri, P. O. Behan, and B.O.R., unpublished observations).

ICA69. Analysis of 48 overlapping 20-mer peptides covering ICA69 showed, in analogy to GAD65 peptides, that ICA69 peptides have relatively higher binding affinities for HLA-DR1 and -DR2 compared with the predisposing HLA-DR3 and -DR4 molecules (Table 3). Binding to HLA-DR3 was either good (38%) or undetectable (56%). One report defined an ICA69 epitope near the NH_2-terminus of the autoantigen

TABLE 2
Binding affinities of GAD65-specific T-cell epitopes

T-cell epitope region	Restriction	Source of T-cells	IC_{50} (µmol/l)	Reference
81–100	HLA-DR1	IDDM patient	0.03	25
161–180	HLA-DR1	Nondiabetic individual	15	26
501–520	HLA-DR1	IDDM patient	2	26
201–220	HLA-DR2	IDDM patient	1.2	24
211–230	HLA-DR2	IDDM patient	1.8	24
191–210	HLA-DR3	Nondiabetic individual	70	*
201–220	HLA-DR3	Nondiabetic individual	70	*
331–350	HLA-DR3	Nondiabetic individual	70	*
491–510	HLA-DR3	Nondiabetic individual	56	*
501–520	HLA-DR3	Nondiabetic individual	70	*
111–130	HLA-DR4	IDDM patient/ tg mice	0.7	24,27
271–290	HLA-DR4	IDDM patient/ tg mice	0.2	24,27,28
351–370	HLA-DR4	tg mice	2.7	27
371–390	HLA-DR4	tg mice	0.8	27
481–500	HLA-DR4	tg mice	4	27
511–530	HLA-DR4	tg mice	5	27
531–550	HLA-DR4	tg mice	10	27
541–560	HLA-DR4	tg mice	10	27,28
551–570	HLA-DR4	IDDM patient/ tg mice	0.7	24,27

*Unpublished observations. tg, transgenic.

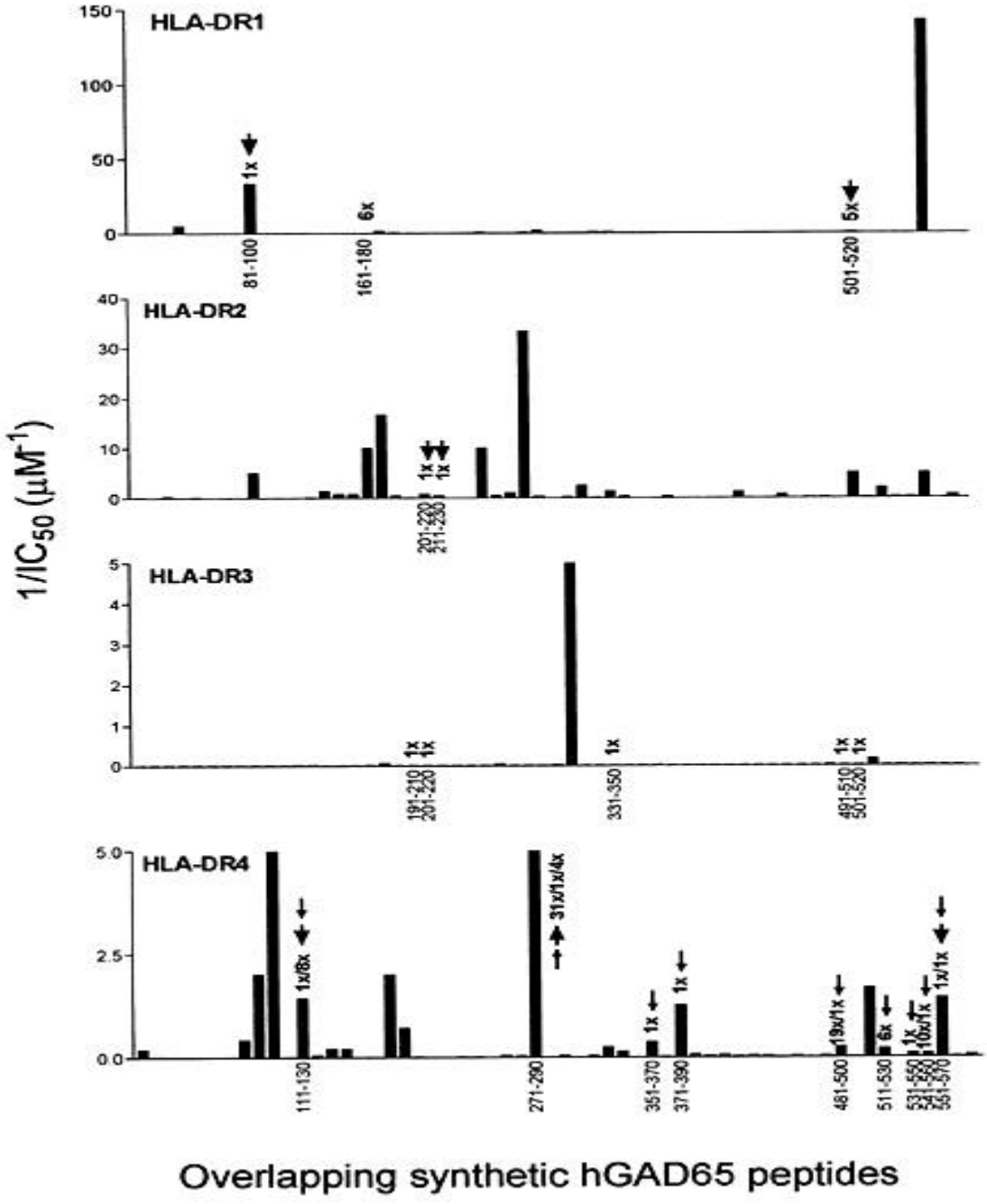

FIG. 1. Binding affinities for HLA-DR1, -DR2, -DR3, and -DR4 of overlapping human GAD65 peptides. Binding is expressed on the *y*-axis as $1/IC_{50}$. IC_{50} is given in micromoles per liter. T-cell epitope regions are indicated on the *x*-axis; the number of different T-cells reported to recognize a peptide region is indicated on top of the corresponding bar. Big arrows indicate T-cell epitopes in IDDM patients (24–26 and N.C.S., G. Duinkerken, R.R.P. De Vries, T. Dyrberg, A. Chaudhuri, P. O. Behan, B.O.R., unpublished observations). Small arrows indicate epitopes in HLA-DR4 transgenic mice immunized with hGAD65 (27,28). The *y*-axis scales differ between the DR alleles.

(p36–47) that was recognized by IDDM patient HLA-DR–restricted T-cells (29). Here, we show that this peptide region of ICA69 has strong binding affinity for HLA-DR1 (neutrally associated with IDDM) and the protective HLA-DR2, but undetectable binding affinity to the predisposing HLA-DR3 and -DR4 molecules.

Insulin and proinsulin. Finally, 15 17-mer peptides, derived from proinsulin and the insulin α- and β-chains, were analyzed for HLA-DR binding. The majority of the (pro)insulin peptides displayed intermediate or low HLA-DR binding affinity. Especially in the case of HLA-DR3, a remarkable lack of high-affinity binding peptides was observed (Table 4). Conversely, HLA-DR2 was associated with the largest fraction of peptides binding with high affinity (47 compared with 7, 0, or 13% for DR1, DR3, and DR4). Most (pro)insulin peptides bound weakly or undetectably to HLA-DR1 (14/15 peptides) and to HLA-DR4 (13/15 peptides).

Validation of HLA-DR peptide binding prediction. To evaluate the predictive value of our peptide binding motif score program, the binding affinity of all overlapping sets of

TABLE 3
HLA-DR binding affinities of ICA69-derived 20-mer peptides (IC_{50} in μmol/l)

	DR1	DR2	DR3	DR4
1–20 MSGHKCSYPWDLQDRYAQDK	>100	>100	>100	10
11–30 DLQDRYAQDKSVVNKMQQRY	70	70	>100	0.07
21–40 SVVNKMQQRYWETKQAFIKA	0.1	0.007	56	0.07
31–50 WETKQAFIKATGKKEDEHVV	0.8	0.008	>100	>100
41–60 TGKKEDEHVVASDADLDAKL	>100	20	22	0.1
51–70 ASDADLDAKLELFHSIQRTC	4	0.001	5	31
61–80 ELFHSIQRTCLDLSKAIVLY	0.8	2	7	0.66
71–90 LDLSKAIVLYQKRICFLSQE	1	50	6	1.0
81–100 QKRICFLSQEENELGKFLRS	2	8	>100	0.5
91–110 ENELGKFLRSQGFQDKTRAG	2	0.5	>100	37
101–120 QGFQDKTRAGKMMQATGKAL	1	0.002	>100	50
111–130 KMMQATGKALCFSSQQRLAL	0.7	0.1	5	0.5
121–140 CFSSQQRLALRNPLCRFHQE	60	0.07	0.2	14
131–150 RNPLCRFHQEVETFRHRAIS	50	0.06	>100	21
141–160 VETFRHRAISDTWLTVNRME	0.08	1	>100	10
151–170 DTWLTVNRMEQCRTEYRGAL	60	5	1.0	5
161–180 QCRTEYRGALLWMKDVSQEL	0.08	0.04	6	0.07
171–190 LWMKDVSQELDPDLYKQMEK	>100	>100	7	10
181–200 DPDLYKQMEKFRKVQTQVRL	0.6	0.008	>100	1
191–210 FRKVQTQVRLAKKNFDKLKM	1	0.02	7	>100
201–220 AKKNFDKLKMDVCQKVDLLG	60	0.1	0.8	8
211–230 DVCQKVDLLGASRCNLLSHM	1	0.2	3	18
221–240 ASRCNLLSHMLATYQTTLLH	0.2	0.1	7	9
231–250 LATYQTTLLHFWEKTSHTMA	10	0.1	>100	60
241–260 FWEKTSHTMAAIHESFKGYQ	1	0.7	>100	7
251–270 AIHESFKGYQPYEFTTLKSL	0.8	0.2	8	0.7
261–280 PYEFTTLKSLQDPMKKLVEK	4	0.3	>100	7
271–290 QDPMKKLVEKEEKKKINQQE	>100	>100	>100	>100
281–300 EEKKKINQQESTDAAVQEPS	>100	70	>100	6.6
291–310 STDAAVQEPSQLISLEEENQ	40	>100	>100	68
301–320 QLISLEEENQRKESSSFKTE	60	6	21	69
311–330 RKESSSFKTEDGKSILSALD	0.3	1	7	0.06
321–340 DGKSILSALDKGSTHTACSG	8	>100	>100	60
331–350 KGSTHTACSGPIDELLDMKS	>100	>100	>100	10
341–360 PIDELLDMKSEEGACLGPVA	4	70	>100	0.5
351–370 EEGACLGPVAGTPEPEGADK	4	>100	>100	>100
361–380 GTPEPEGADKDDLLLLSEIF	50	>100	>100	96
371–390 DDLLLLSEIFNASSLEEGEF	0.8	70	>100	0.07
381–400 NASSLEEGEFSKEWAAVFGD	7	0.8	>100	10
391–410 SKEWAAVFGDGQVKEPVPTM	40	8	115	51
401–420 GQVKEPVPTMALGEPDPKAQ	7	7	>100	16
411–430 ALGEPDPKAQTGSGFLPSQL	>100	>100	>100	>100
421–440 TGSGFLPSQLLDQNMKDLQA	7	>100	50	7
431–450 LDQNMKDLQASLQEPAKAAS	7	>100	>100	14
441–460 SLQEPAKAASDLTAWFSLFA	5	70	53	7
451–470 DLTAWFSLFADLDPLSNPDA	6	>100	0.1	0.07
461–480 DLDPLSNPDAVGKTDKEHEL	>100	>100	>100	60
471–490 VGKTDKEHELTDKEHELLNA	>100	>100	>100	65

peptides was correlated to the occurrence of the different DR motifs (Table 5). The prediction of high-affinity HLA-DR binding peptides proved to be very accurate. In the case of GAD65 and ICA69, for which the number of peptides tested was much larger than for (pro)insulin, a strong correlation was found between the presence of the HLA-DR peptide binding motifs and peptide binding affinity to purified HLA-DR molecules. For DR3 and DR4, in particular, 100 and 94% (GAD65) or 100 and 91% (ICA69), respectively, of the high-affinity binders were predicted. Furthermore, the number of false-positive predicted binding peptides was low. The finding that some nonbinding peptides that contain a HLA-DR binding motif are unable to bind to that HLA-DR may be due to flanking residues (34) that disturb binding of the anchor residues.

DISCUSSION

We here describe the binding affinity of three complete sets of overlapping synthetic peptides of candidate islet autoantigens (GAD65, ICA69, and (pro)insulin) to the IDDM-susceptible alleles HLA-DR3 and -DR4, the protective allele DR2, and the neutral allele DR1. The HLA binding affinities of these peptides correspond well with their

TABLE 4
HLA-DR binding affinities of (pro)insulin-derived 17-mer peptides (IC_{50} in μmol/l)

	DR1	DR2	DR3	DR4
Insulin α-chain				
1–17 GIVEQCCTSICSLYQLE	70	14	70	4
6–21 CCTSICSLYQLENYCN	>100	65	70	24
Insulin β-chain				
1–17 FVNQHLCGSHLVEALYL	5	>100	29	>100
6–22 LCGSHLVEALYLVCGER	>100	>100	61	>100
11–27 LVEALYLVCGERGFFYT	>100	2	50	>100
16–30 YLVCGERGFFYTPKT	>100	6	21	>100
(Pro)insulin				
B20–C4 GERGFFYTPKTRREAED	>100	9	70	>100
B25–C9 FYTPKTRREAEDLQVGQ	>100	>100	70	70
B30–C14 TRREAEDLQVGQVELGG	>100	6	49	40
C3–C19 EDLQVGQVELGGGPGAG	>100	>100	>100	>100
C8–C24 GQVELGGGPGAGSLQPL	>100	>100	>100	>100
C13–C29 GGGPGAGSLQPLALEGS	>100	>100	>100	33
C18–A1 AGSLQPLALEGSLQKRG	24	0.8	70	0.7
C23–A6 PLALEGSLQKRGIVEQC	65	0.4	>100	70
C28–A11 GSLQKRGIVEQCCTSIC	65	2	50	70

(auto)antigenicity as determined in human T-cells or HLA-DR transgenic mice. The high binding affinity of most reported T-cell epitopes and the fact that T-cells reactive with such peptides are found in the periphery may imply that these T-cells are not negatively selected during thymic education. However, it is conceivable that GAD65 is not exposed during thymic education because of its ectopic and low expression in peripheral tissues as pancreatic islets and neurons. In the case of HLA transgenic mice, it may be possible that the immunization with human GAD65 triggered outgrowth of unprimed T-cells that would not have been activated by endogenous GAD65 in vivo (27,28). Moreover, human GAD65 is a xenogeneic antigen in HLA-DR transgenic mice, which might well explain outgrowth of T-cells that had not been eliminated in the thymus (31). An intriguing observation is the low binding affinity for DR3 of GAD65-derived T-cell epitopes recognized in nondiabetic subjects. However, it is in line with the absence of DR3-restricted T-cell epitopes in IDDM (Tables 1 and 2, Fig. 1). Such binding characteristics may imply that this type of reactivity is not pathogenic or that such T-cells are not primed in vivo.

The ICA69 epitope (p36–47) located at the NH_2-terminus and recognized by IDDM patient T-cells (29) strongly binds to HLA-DR1 (neutrally associated with IDDM) and the protective HLA-DR2, but not to the predisposing HLA-DR3 and -DR4 molecules. The suggestion that high-affinity binding of self-peptide may trigger T-cell anergy (29) is therefore conceivable for HLA-

TABLE 5
HLA-DR binding peptides

	Good binders		Intermediate binders		Nonbinders	
	Observed	Predicted	Observed	Predicted	Observed	Predicted
GAD65						
HLA-DR1	27	19 (70)	18	7 (39)	13	4 (30)
HLA-DR2	39	23 (59)	11	4 (36)	8	3 (37)
HLA-DR3	2	2 (100)	19	18 (95)	37	19 (51)
HLA-DR4	18	17 (94)	24	13 (54)	16	7 (44)
ICA69						
HLA-DR1	29	15 (52)	10	5 (50)	9	1 (11)
HLA-DR2	27	15 (56)	7	5 (71)	14	4 (28)
HLA-DR3	18	18 (100)	3	1 (33)	27	14 (52)
HLA-DR4	22	20 (91)	21	18 (86)	5	2 (40)
(Pro)insulin						
HLA-DR1	1	0	3	0	11	2 (18)
HLA-DR2	7	5 (71)	2	2 (100)	6	1 (17)
HLA-DR3	0	0	11	5 (45)	4	1 (25)
HLA-DR4	2	2 (100)	6	5 (83)	7	3 (43)

Data are n or n (%). According to their IC_{50}, the peptides were divided into good binders (<10 μmol/l), intermediate-poor binders (10–100 μmol/l), and nonbinders (>100 μmol/l). Total number of peptides tested: GAD65: n = 58; ICA69: n = 48; (pro)insulin: n = 15.

DR1 and -DR2. For DR3 and DR4, the equivalent self-peptide has a very low binding affinity (27,28). However, this low binding affinity may cause positive selection of T-cells reactive to these self-peptides, which may ultimately lead to autoreactivity.

A remarkable observation is the high binding affinity of (pro)insulin peptides to the "protective" HLA-DR2 (Table 4). These peptides showed weak affinity significantly more often than high binding affinity for the IDDM-susceptibility associated alleles HLA-DR3 and -DR4. Even though the negative association of HLA-DRB1*1501 with development of IDDM can be explained through linkage of this allele with, e.g., HLA-DQB1*0602, it could also be argued, based on our data, that naturally processed self-peptides with high HLA-DR binding affinity can cause elimination of T-cells reactive with this self-protein. Indeed, insulin is produced and expressed in the thymus (32) in contrast to GAD65 and ICA69. Consequently, this scenario predicts that HLA-DR2–restricted T-cell reactivity to (pro)insulin peptides will be a rare event.

The association of HLA-DR4 with insulin autoimmune syndrome cannot readily be explained by our present data. However, few peptides induce tolerance through thymic selection because peptides binding with high affinity to HLA-DR4 are rare (13%), whereas a relatively high fraction of peptides displayed intermediate or weak binding affinity for HLA-DR4, thereby conceivably increasing the risk of positive thymic selection and subsequent autoimmunity.

It has been proposed that molecular mimicry between GAD65 and the coxsackie B4 viral protein P2C plays a role in the pathogenesis of IDDM (10). In this model, infection by coxsackievirus would result in activation of P2C-specific T-cells reactive to peptides binding with high affinity to HLA molecules. Our data indicate that the homologous regions of P2C and GAD65 (the 20-mers P2C p28–47 and GAD65 p243–262) bind well to HLA-DR molecules that are either neutral (DR1) or dominantly negatively (DR2) genetically associated with IDDM. In contrast, these peptides bound with very weak affinity to the IDDM-susceptible alleles DR3 and DR4 (Table 1). Shorter peptides, the 12-mers P2C p34–45 and GAD65 p49–60, still bound to DR3, whereas no appreciable DR4 binding of this minimal epitope covering the PEVKEK region of GAD65 was detected (30).

Extrapolation of peptide–HLA-DR binding to the pathogenesis of IDDM should be done with care. First, the genetic predisposition of HLA-DR alleles with development of IDDM may be secondary or additive to the predisposition imposed by HLA-DQ alleles (16) in linkage disequilibrium with HLA-DR. Second, although the minimal length of HLA-DR binding epitopes (8–10 amino acids) has been met by using 20-mers with a 10 amino acid overlap for HLA-DR binding analysis—which reduces the risk that actual T-cell epitopes are missed in this peptide series—binding affinity of naturally processed equivalent peptides could still be influenced by flanking residues (33).

The prediction of binding affinity based on our peptide binding motif score was especially accurate for the good (IC_{50} values < 10 μM) and intermediate HLA-DR binding peptides (Table 5). Strikingly, for GAD65 and ICA69, the HLA-DR3 peptide binding motif (20) predicted all high-affinity binders. Also, the HLA-DR4 peptide binding motif (23) predicted most (GAD65, 94%; ICA69, 91%) or all [(pro)insulin] peptides binding to this allele. On the other hand, all motifs predicted only 52% or less of the nonbinding peptides. Thus, these HLA-DR motifs and our HLA class II peptide binding motif score program provide a useful tool for the selection of peptides that bind with high affinity to HLA-DR.

In conclusion, our data show that prediction of peptide binding to HLA-DR molecules and of immunogenic potential of candidate islet autoantigens is feasible, because of the strong correlation between antigenicity and HLA-DR binding affinity of GAD65 peptides. However, other mechanisms—such as peptide processing and stability of HLA-DR–peptide complexes (34)—are likely to play a role, as well. Furthermore, negative thymic selection of (pro)insulin peptides, caused by high-affinity HLA-DR2 binding, may explain the lack of autoreactivity to (pro)insulin in HLA-DR2+ individuals.

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Author Queries (please see Q in margin and underlined text)

Tables must be called out in numerical order. I have renumbered Tables 1 through 4. Table 4 becomes Table 1 because it is cited first; Table 1 is 2; Table 2 is 3; Table 3 is 4. Table 5 is unchanged. However, a callout for Table 3 (formerly Table 2) is missing. Please add one in text. Also, please check throughout the text and make sure tables are called out correctly.>

Q1: Please spell out CHAPS.>
Q2: Please give all authors' names and initials for the Schloot unpublished observations.>
Q3: Please give the authors' names for the unpublished observations cited in the legend to Fig. 1.>
Q4: In some places you have P2C and in others you have p2C. Are these different things, or should the capitalization be standardized to one or the other?>
Q5: Did aa stand for amino acids? If not, please correct.>
Q6: I'm not sure what you mean by "good" HLA-DR binding peptides in the sentence beginning "The prediction of…" Can you clarify a bit what you mean by that usage of the word "good"?>
Q7: Please spell out "CEC" and "NWO-CZ-SO."
Q8: Please define IC_{50}.

Ref 17: "Bouwes Bavinck JN" correct as is?