

# T Cell Receptor Variable Regions in Diabetes Bind to Each Other, to Insulin, Glucagon or Insulin Receptor, and to Their Antibodies

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**Abstract:** Our objective is to elucidate the nature of the autoimmune dysregulation in diabetes through the antigen specificity of the T-cell receptor (TCR) sequences generated by patients with type 1 diabetes mellitus (T1DM). Previously we demonstrated that TCR from T1DM patients and NOD mice mimic insulin, glucagon and their receptors. We hypothesize that these TCR will bind to each other (as insulin and glucagon do to their receptors) and also be targets of anti-insulin and anti-glucagon antibodies. The hypervariable regions of multiple TCR from three patients were synthesized and their binding specificities determined using UV spectroscopy. ELISA was used to determine whether these TCR were recognized by anti-insulin and anti-glucagon antibodies. Each patient produced TCR that recognized insulin, glucagon and the insulin receptor (IR). These TCR also recognized each other as complementary (possibly idiotype-antiidiotype) pairs. In addition, each TCR peptide was recognized with nanomolar affinity as an antigen by an antibody against insulin, glucagon, and/or IR. Finally, each of the antibodies against insulin, glucagon and IR formed a complementary antibody (or idiotype-antiidiotype) pair with another antibody involved in the disease, again at nanomolar affinities. Every possible expression of complementarity (or idiotype-antiidiotype cross-reactivity) involving TCRs and antibodies was manifested by each patient. Two interpretations of these observations are offered. One, following Marcheloni, is that TCR-antibody complementarity is a mechanism for down-regulating the autoimmune process to re-establish tolerance to self-antigens. A non-exclusive alternative is that the trigger for autoimmunity is antigenic complementarity, which results in the production of complementary TCR and antibodies that appear to have idiotype-antiidiotype relationships among themselves.

**Keywords:** T-cell receptor cross-reactivity, TCR-TCR antiidiotypy, antibody-TCR antiidiotypy, antibody-antibody antiidiotypy, diabetes, islet cell antigen, PTP-IA-2, insulin, insulin receptor, glucagon, glucagon receptor, insulin antibody, insulin receptor antibody, glucagon antibody, antigenic complementarity, complementary antigens, complementary antibodies, complementary TCR, complementary BCR, molecular mimicry.

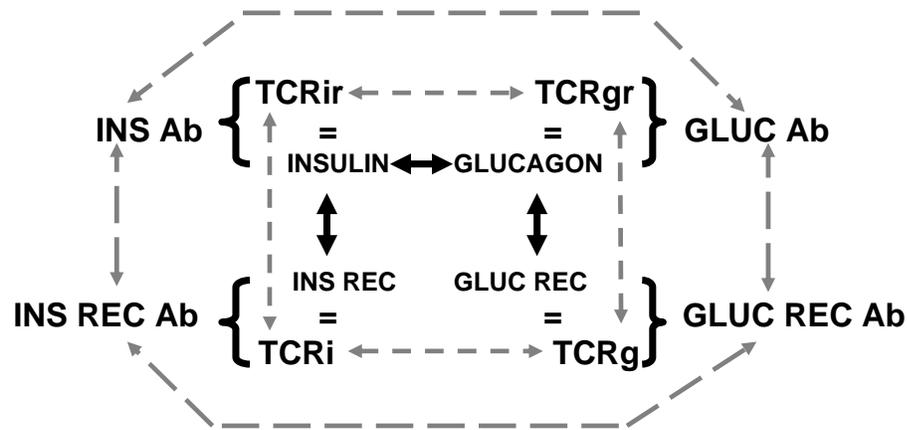
## INTRODUCTION

Insulin is a major target of the autoimmunity involved in type 1 diabetes mellitus (T1DM) [1-9] as is the islet cell antigen 512 (receptor-type tyrosine protein phosphatase-like N protein, PTP-IA-2) [10-13] and in many cases the insulin receptor [14-18]. The cause and nature of the immune dysregulation that results from the targeting of insulin and islet-cell antigens is still mysterious. One clue may reside in the specificity of the T cell receptors (TCR) generated during the disease. The hypervariable (V $\beta$ /D/J $\beta$ ) regions of T-cell receptors (TCR) from patients with T1DM and from the NOD mouse model of diabetes have been sequenced previously by several investigators [4, 19-24]. The majority of these human diabetic and NOD mouse TCR sequences mimic insulin, glucagon, or their receptors (a selection of these is summarized here in Table 1) [25]. These observations

suggest a series of surprising consequences that could have serious immunological implications for diabetics.

Since insulin is complementary to its receptor, and glucagon to its receptor, some diabetic TCR may correspondingly be complementary to each other and might therefore bind to each other as they would to antigenic targets. In other words, these TCR might act like idiotype-antiidiotype pairs. Other complementarities have also been reported. The insulin receptor has multiple regions that mimic insulin and glucagon, and the glucagon receptor has multiple regions that mimic glucagon and insulin [26-28], so that these receptors might also be targets of diabetic TCR. In addition, insulin and glucagon are complementary to each other and produce primary antibodies that are complementary, and so act like idiotype-antiidiotype pairs [29]. In consequence, TCR against insulin and glucagon might also be complementary to each other, and appear to act like idiotype-antiidiotype pairs. Patients with diabetes are also characterized by having *antibodies* against insulin [1-9], the insulin receptor [14-18] and to glucagon [30-31], so that these *antibodies* may target TCR mimicking insulin and glucagon or their receptors (Fig. 1).

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**Fig. (1). Possible interactions between Figure Captions.**

**TCR and antigens in T1DM.** Graphic illustration of known (black lines) and posited (gray lines) complementarities that exist between insulin, glucagon, their receptors; antibodies against these hormones and their receptors; and TCR sequences from T1DM patients. Insulin is complementary to its receptor, as is glucagon to its receptor. Antibodies to each hormone and each receptor are also complementary. In addition, insulin is complementary to glucagon so that the two hormones bind to each other and their antibodies are complementary so they act like an idiotype-antiidiotype pair [29]. The situation is complicated by the fact that the insulin receptor contains multiple regions that mimic insulin itself, and these insulin-like regions are associated with insulin binding [26-28]. Similarly, the glucagon receptor has glucagon-like sequences that appear in glucagon-binding regions of the receptor [26-27]. Thus, it can be predicted that antibody against insulin may bind to the insulin receptor and that antibody against glucagon may bind to the glucagon receptor (dotted gray lines). The set of possible antigenic cross-reactions is further complicated by the fact that, as described in Table 1, the TCR from T1DM patients used in this study are similar to insulin (TCRi), glucagon (TCRg), insulin receptor (TCRir) or glucagon receptor (TCRgr), which is indicated by the equals signs [25, 29]. In consequence, it is possible that: 1) TCR within a T1DM patient bind directly to insulin, glucagon, and/or their receptors (dotted gray lines); 2) that these TCR mimic the binding affinities of insulin, glucagon, and/or their receptors by *binding to each other*, i.e., they are complementary and act like idiotype-antiidiotype pairs (dotted gray lines); 3) that antibodies against insulin, glucagon and their receptors will recognize the TCR that mimic these peptides and proteins (dotted gray lines); and 4) that the antibodies against insulin, glucagon, and their receptors will interact in all their possible combinations to produce a network of idiotype-antiidiotype pairs (dotted gray lines). In sum, the sets of complementarities found among the hormones and their receptors may be mimicked by the sets of complementarities found among the TCR that mimic them and antibody reactivity to these TCR. Such complementarities should be reflected in multiple types of cross-reactivity and complementary networks within individual T1DM patients. Note that the islet cell antigen 512 (PTP-IA-2), which is known to be a target of autoimmunity in T1DM [10-13] is highly homologous to insulin and the insulin receptor (Table 1), so that TCR are also likely to recognize it as an antigen. PTP-IA-2 may therefore be substituted in each place that insulin and insulin receptor appear in this diagram as a means of making predictions about its cross-reactivities with T1DM-generated TCR. See also Figs 7C and 7D.

And finally, we demonstrate here that the islet cell autoantigen PTP-IA-2 [10-13] has significant homologies to both insulin and to the insulin receptor (Table 1) so that the various cross-reactivities described above for insulin and the insulin receptor may also apply to PTP-IA-2. These predictions would extend the concept of a network of complementary interactions (perhaps described by an idiotype-antiidiotype network) to interactions not only between antibodies, but also between pairs of TCRs as well as between antibody-TCR pairs in diabetes. The purpose of this paper is to report the results of experimental tests of these possibilities.

## MATERIALS AND METHODS

### Homology Searches

A search for possible homologies between human insulin (P01308), the insulin receptor (P06213), and PTP-IA-2 (islet cell antigen 512) (Q16849) was carried out at <http://www.expasy.ch> using LALIGN [32] using PAM250 and BLOSUM 80 search algorithms with gap penalties set on default parameters. Significance of homologies was determined by two criteria: at least 5 identities in a stretch of 10 amino acids and/or an E value greater than 35 (a measure

of the probability of  $p < 0.01$  that equivalent homologies would be found in a random search of 10,000 protein sequences of equivalent length). The comparison of the insulin receptor with PTP-IA-2 was carried out in two ways: 1) using the entire insulin receptor sequence and 2) using two specific sequences from the insulin receptor that have been identified as insulin-binding regions [28] (see Table 1 for sequences). These two insulin receptor peptides, IR 105-118 and IR 897-915 (SwissProt numbering from P06213), were synthesized and purified to at least 98% purity by the Mass Spectroscopy, Synthesis and Sequencing Facility of the Department of Biochemistry of Michigan State University and used as part of the studies described below to determine TCR affinity.

### Synthesis of TCR sequences

Eight hypervariable V $\beta$  regions from TCR of three T1DM patients and seven hypervariable V $\beta$  from TCR of patients infected with HIV were synthesized and purified to at least 98% purity by the Mass Spectroscopy, Synthesis and Sequencing Facility of the Department of Biochemistry of Michigan State University (Table 2). The eight diabetic TCR were chosen because they represented at least three different

**Table 1. Homologies between Insulin or Insulin Receptor Peptides and PTP-IA-2 (Islet Cell Antigen 512). PTP-IA-2 is a Major Target of Autoimmunity in T1DM. It has Many Significant Homologies with Insulin (see Table 2) and with the Two Insulin Receptor Peptides (95-113 and 897-916) that were Tested for Recognition by TCR (See Tables 1 and 4). Because of these Multiple Homologies, it is Likely that at Least Some of the TCR that Recognize Insulin and IR Peptides Will Recognize PTP-IA-2 as Well. Notably, Honeyman, *et al.* [13] have Demonstrated that Patients with T1DM Produce TCR Reactive to Several Cytoplasmic Regions of PTP-IA-2, in Particular 847-856 and 941-959, Both of which Appear in this Table. Honeyman, *et al.*[13] did not, however, test any of the Extracellular Regions of the Protein, which Encompasses Amino Acids 35-575, in which most of the Homologies Listed in this Table Appear. Further Research into these Homologies is Therefore Warranted**

<b>PTP-IA-2 951-959 (Q16849)</b>	<b>GLVRSKDQF</b>
	/
<b>Insulin Receptor 95-113 (P06212)</b>	<b>GLESLKD LFPNLTVIRGSR</b>
	: :     :
<b>PTP-IA-2 941-952 (Q16849)</b>	<b>LEHVRDQRPGI</b>
<b>PTP-IA-2 598-615 (Q16849)</b>	<b>LCV- RQHARQQDKERLAAL</b>
	:   \ : : :     :
<b>Insulin Receptor 897-916 (P06212)</b>	<b>HLCVSRKHFA L ERGCRLRGL</b>
	:
<b>PTP-IA-2 110-117 (Q16849)</b>	<b>MER I PRLR</b>
<b>PTP-IA-2 16-59 (Q16849)</b>	<b>LRLLLCLLLLSSRPGGCSAVSAHGCLFDRRLCSHL –EVC IQDGLF</b>
	:        :     : : / : : : :   : :   :      :   :
<b>Insulin 5-49 (P01308)</b>	<b>MRLPLLALLALWGPDPAAAFVNQHLGSHLVEALYLVCGERGFF</b>
	:   :   : : : :   /   :    :   :
<b>PTP-IA-2 612-636 (Q16849)</b>	<b>LAALGPEGAHGDTTFEYQDLCRQHM</b>
<b>PTP-IA-2 839-856 (Q16849)</b>	<b>LVSEH IWCEDFLVRSFYI</b>
	:   : :   :   :     : :
<b>Insulin 25-41 (P01308)</b>	<b>FVNQHL- CGSHLVEALYL</b>
<b>PTP-IA-2 147-179 (Q16849)</b>	<b>TGSAPAAQHRLPQPPVGKGGAGASSLSPLQAE</b>
	: : : : :   / :   :     : : :     :     / :
<b>Insulin 51-83 (P01308)</b>	<b>TPKTRREAEDLQVGQVELGGGPGAGSLQPLALE</b>
	:   :         : :     :
<b>PTP-IA-2 224-236 (Q16849)</b>	<b>EGSPGMVSVGPLP</b>
<b>PTP-IA-2 165-179 (Q16849)</b>	<b>GGAGASSLSPLQAE</b>
	:     :     :     / :
<b>Insulin 53-83 (P01308)</b>	<b>KTRREAED -LQVGQVELGGGPGAGS– LQPLALE</b>
	: :     : :     :   : :   : : :
<b>PTP-IA-2 538-565 (Q16849)</b>	<b>KSELEAQTGLQ I LQTGVGQRE EAAA - VLP</b>

sequences from each of three patients. TCR 1, 2 and 4 were from one patient [4]; TCR 8, 9, and 10 from a second patient [4] ; and TCR K2.4, K2.12, and K2.16 from a third [23]. TCR K2.12 and TCR 2 were highly homologous, so only the TCR 2 sequence was synthesized. Similarly TCR K2.16 and TCR 4 were highly homologous, so only TCR 4 was synthesized. Three of the TCR shared a common similarity (4, 8 and 9) so a single consensus sequence was synthesized. Notably, TCR K2.12 and TCR 10 are almost identical to TCR from multiple NOD mice [20, 23, 25] and similarities to TCR 1, 2, 8, 9, K2.4, and K2.16 are also evident with NOD TCR [25]. Thus, the interactions between the TCR sequences synthesized here are likely to be characteristic of diabetic and NOD TCR from multiple patients and diabetic animals. The HIV-related TCR were used as controls for this study.

**UV Spectroscopy Study of TCR-Antigen Binding**

Four sets of experimental tests were run. First, ultraviolet (UV) spectroscopy was used to determine whether the TCR sequences listed in Table 1 bind to insulin (Sigma-Aldrich), glucagon (Sigma-Aldrich), or either of the two peptides IR 105-118 and IR 897-915, derived from the insulin receptor (IR) as described above (see Table 1 for sequences). These hormone and IR peptides were dissolved as solutions of ca. 0.5 mM in pH 7.4 phosphate buffer (Fisher Scientific) and 50 μM solutions were then made for use in spectroscopy. The TCR peptides were dissolved in the same buffer at 0.5 mM and then serially diluted eight times by thirds. 100 μl of each TCR concentration plus 100 μl of buffer; mixtures of 100 μl of each hormone or IR peptide with 100 μl of buffer; and 100 μl of each hormone or IR peptide with 100 μl of

each TCR concentration were made in a crystal 96-well plate and a complete spectrum from 190 nm to 250 nm of each well recorded on a Spectromax Plus spectrophotometer. Data were collected using SoftMax Pro 4.0 software. Every combination was done in duplicate and the results averaged. These curves were examined for the absorbance at which the greatest spectral shifts occurred (usually 205 nm). The absorbance of a mixture of the hormone or IR peptide with TCR peptide was compared with the sum of the absorbances of the hormone or IR peptide (plus buffer) and TCR (plus buffer) and the differences plotted as a function of the concentration of the TCR peptide. A flat line resulted if there was no binding and an "S"-shaped curve if binding was evident. Binding constants were determined from the inflection point of the curves. In general, with the range of concentrations of peptides used here, this method is able to determine binding constants between about 100 nM and 1 mM.

### UV Spectroscopy Study of TCR-TCR Binding

UV spectroscopy was also used to determine whether any of the TCR bound to each other and the same protocol was used as above, holding one TCR at a constant concentration of about 50  $\mu$ M and varying the concentration of the other by thirds starting at 0.5 mM. All possible combinations of the TCR were tested holding each one constant and varying the others.

### ELISA Study of Antibody-TCR Binding

A third set of experiments utilized an enzyme-linked immunoadsorption assay (ELISA) to test the ability of antibodies against human insulin (guinea pig, Biodesign K10264C), human glucagon (rabbit, Millipore, AB932), the alpha protein of the insulin receptor (rabbit, Biodesign, K54244R), the beta protein of the insulin receptor (mouse, Biodesign, K54241M), or antibody against the glucagon receptor (rabbit, Santa Cruz Biotechnology, sc-66912) to recognize any of the diabetic TCR peptides. Each TCR peptide was dissolved in buffer to produce a 50  $\mu$ M stock solution and 100  $\mu$ l were then added to an ELISA plate (Costar), incubated for an hour, and then triply washed with a 1% solution of TWEEN in phosphate buffer. 200  $\mu$ l of a 2% polyvinylalcohol (PVA) blocking agent in phosphate buffer was added to each well, incubated for an hour, and triply washed. 100  $\mu$ l of a 1:100 dilution of antibody was then added to each well, incubated for an hour, and triply washed. 100  $\mu$ l of a horse-radish-peroxidase (HRP) linked secondary antibody appropriate to the species of the test antibody (Chemicon) was then added to each well and incubated for an hour before being washed out. Finally, 100  $\mu$ l of ABTS single reagent (Chemicon) was added to each well, incubated for 30 minutes, and the plate read at 405 nm in a Spectromax scanning spectrometer. All combinations and controls were run in duplicate and the resulting values averaged.

### ELISA Study of Idiotype-Antiidiotype Antibody Binding

Finally, the antibodies listed above were tested for binding to each other (i.e., for their ability form idiotype-antiidiotype pairs) using a double-antibody (DA) ELISA protocol [29, 33]. DA-ELISA is performed just as a standard ELISA is, but the antigen is replaced with a primary anti-

body (plated in dilutions from 1/30 through 1/1,000,000 by thirds in pH 7.4 phosphate buffer). The test antibody must be from a species other than that used as the primary antibody and is applied at 1/100 dilution. An enzyme-linked antibody against the test antibody species is then added (1:1000 dilution) and used as a reporter of the test antibody binding to the primary antibody. The primary and test antibodies must be from different species in order for the reporter antibody to be able to differentiate between them. Since many of the antibodies used above were derived from rabbits, an additional monoclonal antibody against the insulin receptor (mouse, Biodesign, E42365M) was substituted for rabbit anti-insulin receptor in some of the DA-ELISA studies in order to increase the number of combinations testable.

### Data Analysis

All data were analyzed and plotted using an Excel spreadsheet program.

### A Note on Insulin Receptor Numbering

All IR sequences (P06213) are provided in the SwissProt numbering system (<http://www.expasy.ch>) that begins with the pre-pro sequence. Many papers on the IR use a numbering system that begins with the edited transcript and are thus have 27 amino acids subtracted from the number as compared with the SwissProt convention.

## RESULTS

### Antigen Homologies.

Many significant homologies were found between human PTP-IA-2 (islet cell antigen 512) and both insulin and the insulin receptor. Table 1 summarizes a few of these. Many more (dozens) were found but only a representative listing is made here. Most of the homologies between PTP-IA-2 and insulin are in the insulin A chain, and these homologies appear scattered throughout the entire islet cell antigen protein. A similarly large number of homologies was found between PTP-IA-2 and the insulin receptor, but only the four most significant of those related directly to the two insulin-receptor peptides (IR 93-113 and IR 897-916) used in the experimental studies carried out here are shown in Table 6. Notably, these homologies are similar to the insulin B chain as well [25].

### TCR-Antigen Binding

Table 2 summarizes the fact that all of the diabetic TCR synthesized for this study mimic either insulin, glucagon or one of their receptors [25]. Table 3 and Fig. (2) show that six of these eight TCR also bind directly to insulin, glucagon, or peptides from the insulin receptor with affinities ranging between 15  $\mu$ M and 500  $\mu$ M. Two of the eight TCR did not bind to any of the peptides tested, but may have affinity for glucagon receptor, which was not tested. Given the fact that insulin itself is a major target of autoimmunity in diabetes, it is notable that five of the TCR recognized insulin as an antigen with affinities in the low to mid-micromolar range and five of the TCR also recognized IR peptides with 100-200  $\mu$ M affinity. It is also worth noting that these binding constants may be significantly higher than real TCR would dis-

play, since real TCR have several degrees less thermodynamic freedom by being bound into membranes than do the freely rotating peptides used in these solution binding experiments. It is likely, therefore, that TCR *in situ* within the membranes of T cells would exhibit higher affinities for glucagon, insulin and the insulin receptor than are reported here.

**TCR-TCR Binding**

The same interpretative caveats apply to the results summarized in Table 4 and Fig. (3), which shows the affinities of the various TCR peptides for each other. Most of the TCR peptides from the diabetic patients bind to other TCR peptides from diabetic patients with low to mid-micromolar affinities as expected from the various complementarities of the proteins they mimic. Most of the TCR therefore bear some complementary (possibly idiotype-antiidiotype) relationship with each other. None of the TCR from diabetic patients bound with measurable affinity to any of the seven TCR sequences from HIV-infected patients.

**Antibody-TCR Binding**

Table 5 and Figs (4 and 5) demonstrate that all of the diabetic TCR peptides were recognized as antigens at low nanomolar affinities by either polyclonal antibody against insulin (six of eight), glucagon (five of eight), or the alpha subunit of the insulin receptor (six of eight). Four of the TCR were recognized to a measurable degree by all three antibodies although some of the binding was nearly micromolar. These data suggest a complex set of complementary (possibly idiotype-antiidiotype) relationships between TCR and antibodies in diabetes. Notably, however, no TCR was recognized by the polyclonal antibodies against the beta subunit of insulin or the glucagon receptor that were utilized in these experiments.

**Antibody-Antibody Binding**

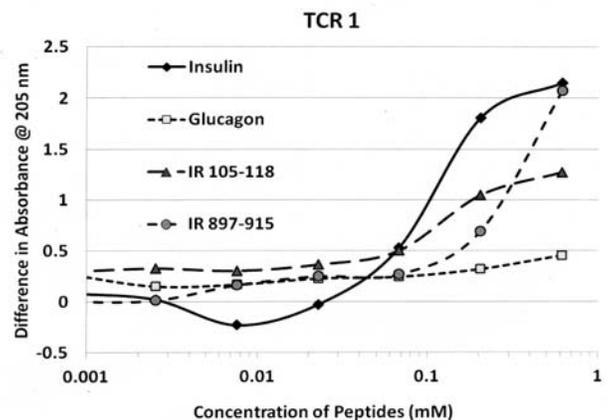
Table 6 summarizes data showing that several of the antibodies used in these experiments recognize each other as complementary (possibly idiotype-antiidiotype) pairs. Polyclonal insulin antibody recognized a monoclonal insulin receptor antibody with mid-picomolar affinity, while polyclonal insulin antibody recognized polyclonal glucagon antibody with nanomolar affinity (see also [29]). Various other mid-nanomolar interactions were also observed between insulin receptor antibodies and either insulin or glucagon receptor antibodies, suggesting further antibody complementarities (Fig 6).

**Multiple Complementary (Possibly Idiotype-Antiidiotype) Pairs In Every T1DM Patient**

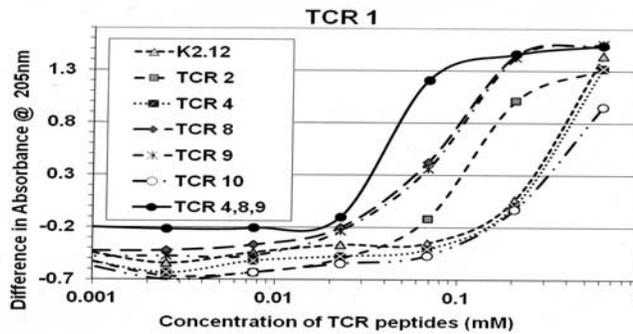
What is most notable about these results is that the TCR from each individual patient from which they were derived (TCRs 1, 2 and 4 for patient 1; TCRs 8, 9, and 10 for patient 2; and TCRs K2.4, K2.12, and K2.12 for patient 3) display at least one complete set of TCR-antigen, TCR-TCR, TCR-antibody, and antibody-antibody interactions. For Patient 1, a female, the interactions were between the TCR peptides and Ins, Ins Rec peptides; most of these TCR peptides acted as complements for each other; and the TCR peptides were

**Table 2.** Similarities between T Cell Receptor (TCR) Sequences and Insulin, the Insulin Receptor, Glucagon, and the Glucagon Receptor. The TCR Designations Refer to the Annotations Used by the Authors who Originally Sequenced them (See Text for Sources). TCR 1, 2, and 4 were Sequenced from one T1DM Patient [4]; TCR 8, 9, and 10 from Another [4]; and K2.4, K2.12, and K2.14 from a Third [23]. Note that Some TCR have More than one Designation Because more Than One Patient had Either Identical or Very Similar Sequences. The Sequence in Bold is that which was Synthesized and Used in the Experiments Described here. Not Shown is the Further Fact that Many of these Sequences are Very Similar to TCR from NOD Mice [25]

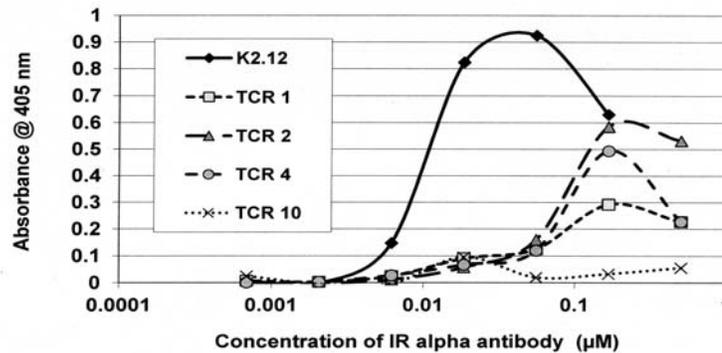
<b>TCR diabetic 1 (GLUC)</b> GLUCAGON 11-21	<b>DSSIYLCVSVEATRAD</b> DYSKYLDSSRA
<b>TCR diabetic 2, K2.16 (GR)</b> GLUC REC 187-199	<b>CSAMYLCASSLAVIRTDQY</b> ASFVLKASSVLVI
<b>TCR diabetic 4, K2.4 (IR)</b> INS REC 927-942	<b>SSLATSGGGSDTQY</b> ATSLAGNGSWTEPTYF
GLUC REC 464-473	SSAETPLAGG
<b>TCR diabetic 8 (Ins, GR)</b> INSULIN 45-51	<b>CASSQVRLAGGGEQ</b> QVEL- GGG
INSULIN 18-27	ALYLVCGERG
<b>TCR diabetic 9 (Ins and GR)</b> GR 440- 457	<b>SSLGQG - -ETEAF</b> SSSPGHGPPSKELQFGRG
<b>TCR diabetic 10 (IR)</b> INS REC 232-245	<b>GVY-CASRNGLNTE</b> GLC- CHSECLG- NCSQ
<b>TCR diabetic K2.12 (IR)</b>	<b>CASSDRLG-NQPQH</b>
GLUC REC 160-169	ALLL ALA ILG
INSULIN 18-26	ALYLVCGERG
<b>TCR diabetic 4,8,9 (Ins, IR, GR)</b> INS REC 893-904	<b>DSALYLCASSLG</b> DEELHLCKSRK



**Fig. (2).** UV spectroscopic study showing that T1DM-derived TCR1 peptide binds to insulin and a peptide from the alpha subunit of the insulin receptor with micromolar affinity. It also binds to glucagon somewhat more weakly, but not to a peptide from the beta subunit of the insulin receptor. Table 3 summarizes data derived from binding studies involving the rest of TCR described in Table 2.



**Fig. (3).** UV spectroscopic study showing that T1DM-derived TCR1 peptide binds to other T1DM-derived TCR peptides. The TCR-TCR affinities vary between low micromolar and unmeasurably small. Table 4 summarizes data derived from binding studies involving every possible combination of the rest of the TCR described in Table 2. Table 4 also shows the results of binding studies of these diabetic TCR to TCR sequences from people with HIV infections. No TCR from an HIV positive individual bound to any of the diabetic TCR tested.



**Fig. (4).** Results of an ELISA study of antibody (rabbit) against the alpha subunit of the insulin receptor (IR) binding to the TCR described in Table 1. The IR antibody recognized TCR K2.12 with low nanomolar affinity, TCR 1, 2, 4 and 8 with mid-nanomolar affinity. Those TCR not shown were not recognized by the antibody at any concentration. Similar data for all other antibody-TCR combinations are summarized in Table 5.

**Table 3.** Binding of TCR to Insulin, Glucagon and Insulin-receptor Derived Peptides. UV Spectroscopic Study of the Binding of TCR from Table 2 to Insulin, Glucagon, and Two Insulin-like Peptides from the Insulin Receptor that have been Shown to have Nanomolar Affinity for Insulin [28]. Binding Constants are Expressed in Micromoles

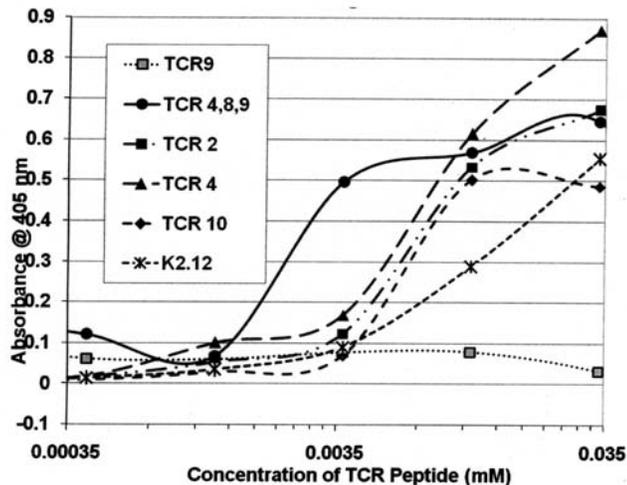
Binding Constants (µM)	Insulin	Glucagon	Ins Rec α 105-118	Ins Rec β 897-915
TCR 1 (Gluc)	80	>10,000	125	300
TCR 2, K2.16 (GR)	>1000	>10,000	>1000	>1000
TCR 4, K2.4 (IR)	15	>1000	>1000	>1000
TCR K2.12 (IR)	75	120	120	> 1000
TCR 8 (Ins, GR)	130	140	120	130
TCR 9 (Ins, GR)	23	140	150	145
TCR 10 (IR)	130	90	110	140
TCR 4,8,9 (Ins, IR, GR)	>1000	>1000	>1000	>1000

recognized by insulin antibody, insulin receptor antibody, and glucagon antibody; and several of these antibodies act as idiotype-antiidiotype pairs. A similar profile characterized patient 2, a male, whose TCR peptides recognized insulin, insulin receptor peptides, and glucagon; bound to each other as complementary pairs, and were recognized by antibody against insulin, insulin receptor, and glucagon, which also interacted as complementary pairs. Finally, Patient 3, another

male, had TCR peptides that recognized insulin, insulin receptor peptides, and glucagons; these TCR interacted as complementary pairs; and were recognized by antibodies against insulin and glucagon antibodies, which are also a complementary pair. In sum, every patient displayed idiotypic recognition of diabetes-related antigens as well as all possible complementary (possibly idiotype-antiidiotype) permutations involving TCR and antibodies.

**Table 4. UV Spectroscopic Study of the Binding of TCR from Table 2 to Each Other and to Some TCR Induced by HIV Infection. The Similarities of Each Peptide to Insulin (INS), Glucagon (GLUC) or their Receptors (IR or GR) are Indicated in the Left-most and right-most Columns. Binding Constants are Expressed in Micromoles**

Binding Constants ( $\mu\text{M}$ )	TCR 1	TCR 2, K2.16	TCR 4, K2.4	TCR 8	TCR 9	TCR 10	TCR 4,8,9 (Ins, IR, GR)
TCR K2.12 (IR)	90	130	250	110	110	110	180
TCR 1 (Gluc)		110	310	70	70	150	33
TCR 2, K2.16 (GR)			230	220	330	120	>10,000
TCR 4, K2.4 (IR)				400	470	110	>1000
TCR 8 (Ins, GR)					400	270	200
TCR 9 (Ins, GR)						>1000	>1000
TCR 10 (IR)							90
HIV TCR 1 CASSELAGGSYNE			>10,000	>10,000	>10,000	>10,000	
HIV TCR 2 CASSERGTNSPL			>10,000	>10,000		>10,000	
HIV TCR 3 CASSGDSRDEQF			>10,000	>10,000		>10,000	
HIV TCR 4 CASSLELAKNI			>10,000	>10,000		>10,000	
HIV TCR 5 CASSLWVTGGEQFF			>10,000	>10,000		>10,000	
HIV TCR 7 CASSLTVSSYNEQ						>10,000	
HIV TCR 8 RCASSSGANV						>10,000	



**Fig. (5). Results of an ELISA study of antibody (guinea pig) against insulin binding to the TCR described in Table 1. The IR antibody recognized TCR 4,8,9 with sub-nanomolar affinity, TCR 2, 4 and 10 with mid-nanomolar affinity. Those TCR not shown were not recognized by the antibody at any concentration. Similar data for all other antibody-TCR combinations are summarized in Table 5.**

**DISCUSSION AND CONCLUSIONS**

**Complementary (Possibly Idiotype-Antiidiotype) Networks in Diabetes**

The current consensus is that T1DM is an autoimmune disease targeted at insulin and the pancreatic beta cells that produce it. One novel finding suggested by our data is that autoimmunity in T1DM may involve intra-immunological targets as well. TCR derived from human diabetic patients

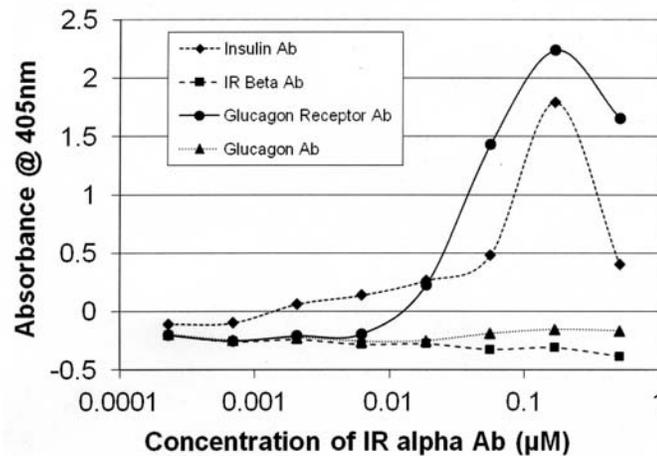
[4, 20], and similar to those from NOD mice [20, 23, 25], recognize insulin, glucagon, and regions of the insulin receptor associated with insulin binding as antigens; these same TCR recognize other TCR from the same patients as antigens; these TCR are, in turn, recognized as antigens by antibodies to insulin, glucagon, and the insulin receptor; and finally these antibodies recognize each other as antigens. In sum, all of the theoretically possible permutations of complementary pairings that were predicted to be possible in Fig.

**Table 5. ELISA Study of Antibody Binding to the TCR Peptides Described in Table 1. The Antibodies Used were Against Insulin, Glucagon, the Alpha Subunit of the Insulin Receptor, the Beta Subunit of the Insulin Receptor, and the Glucagon Receptor. Binding Constants are Expressed in Nanomoles**

Binding Constants (nM)	Insulin Ab	Glucagon Ab	Ins Rec $\alpha$ Ab	Ins Rec $\beta$ Ab	Glucagon Rec Ab
TCR 1 (Gluc)	220	110	24	>1000	>1000
TCR 2, K2.16 (GR)	41	63	28	>1000	>1000
TCR 4, K2.4 (IR)	55	99	30	>1000	>1000
TCR K2.12 (IR)	550	>1000	3.8	>1000	>1000
TCR 8 (Ins, GR)	>1000	>1000	88	>1000	>1000
TCR 9 (Ins, GR)	>1000	2.2	>1000	>1000	>1000
TCR 10 (IR)	33	>1000	>1000	>1000	>1000
TCR 4,8,9 (Ins, IR, GR)	12	5.0	300	>1000	>1000

**Table 6. Double Antibody ELISA Study of Idiotype-antiidiotype Interactions Among Antibodies Against Insulin, Glucagon, and their Receptors. These are the Same Antibodies as those Used in Deriving the Data Described in Table 4, with the Exception that a Monoclonal Antibody against Insulin Receptor was Also Employed so as to Expand the Range of Possible Antibody Combinations (See Text). These Data Expand Upon Those Previously Reported by Root-Bernstein and Dobbstein [29]**

Binding Constants (nM)	Insulin Ab (Guinea Pig)	Ins Rec $\alpha$ Ab (Rabbit)	Ins Rec $\beta$ MAb	Glucagon Rec Ab (Rabbit)	Glucagon Ab (Rabbit)
Insulin Ab (Guinea Pig)		440	38	88	1.1
Insulin Receptor MAb	0.2	>1000	>1000	>1000	>1000
Insulin Receptor $\alpha$ Ab (Rabbit)	127		>1000	83	>1000



**Fig. (6).** Double antibody ELISA experiment showing that rabbit polyclonal antibody against the alpha subunit of the insulin receptor binds to antibody against insulin and rabbit polyclonal antibody against the glucagon receptor but not to antibody against the beta subunit of the insulin receptor or to antibody against glucagon. These results demonstrate that, just as insulin and glucagon are complementary [25, 29], as are their antibodies [25, 29], so are key antigenic determinants of their receptors (see Table 6).

(1) are manifested by the TCR and antibodies tested here. It will be interesting to see if additional manifestations of these interactions occur with specific peptides derived from the glucagon receptor and with other glucagon receptor antibodies as well.

We note that since performing the TCR syntheses, the TCR sequences of another T1DM patient has also been published [33]. Three of the seven sequences from this additional patient are similar to the sequences analyzed in this study (Table 7) and to those of NOD mice [4, 20, 23, 25]. Thus, it appears that a very high degree of constraint limits

**Table 7. Similarities between Three of the Seven V $\beta$  TCR Sequences Expanded from a Male Diabetic Patient in a Study Made Since the Experiments Performed here were Completed [33]. These Sequences Confirm that Similar TCR Sequences Appear to Characterize the Immune Response to T1DM in Both Human Patients and Mice [4, 20, 23, 25]**

V $\beta$ 7	CASSQVAGAGTGELFF
<b>TCR 8 (ins, GR)</b>	<b>CASSQVRLAGGGEQFF</b>
V $\beta$ 1	CASSVSTT - - - DTQYF
<b>TCR4 , K2.4 (IR)</b>	<b>CASSLATSGGGSDTQYF</b>
V $\beta$ 11	CA-SSDP-GTQETQYF
<b>TCR K2.12 (IR)</b>	<b>CASSDRLGNQ -PQHF</b>

the TCR expression resulting in T1DM. The antigen mimicry expressed in the TCR by this additional T1DM patient suggest that he had TCR against the IR, insulin and perhaps the glucagon receptor.

### **Insulin, Insulin Receptor, Glucagon and PTP-IA-2 as Integrated Targets of T1DM**

The data confirm that both insulin [1-9] and the insulin receptor [14-19] can be targets of autoimmunity in diabetes. The high degree of homology between PTP-IA-2 (islet cell autoantigen 512) and both insulin and the insulin receptor (Table 1) suggest that the TCR tested in this study are likely to recognize PTP-IA-2 as a possible antigen as well. Thus, TCR cross-reactivity and mimicry of autoantigens in T1DM may extend to islet cell targets other than insulin, including both IR and PTP-IA-2, in diabetes.

It is important to stress that while it is often asserted that IR autoantibodies result from an antiidiotypic response to insulin autoantibodies [15, 17, 34-37], a number of studies have documented T1DM patients who presented first with autoantibodies to IR that later gave rise to autoantibodies to insulin [16, 18] and patients in which both sets of antibodies were present at diagnosis and prior to the initiation of insulin treatment [14, 38]. Thus, it is possible for autoantibodies against IR to emerge before those against insulin, with them, or after them, making it difficult or impossible to conclude which, if either, is the antiidiotypic to the other.

Since IR are expressed on most, if not all cells, there is also the possibility that anti-IR autoimmunity may extend beyond the pancreas. RNA expression patterns of the IR demonstrate that in addition to pancreas, IR are expressed at very high levels (approximately 50 fold higher than the levels found in most tissues) in ovaries, placenta and the adrenal cortex, and at about five or more fold typical levels in a variety of lymphocyte precursors, kidney, prostate and seminiferous tubules, retina and cerebellum [39]. These tissues and organs are, notably, those that are often most adversely affected during the progression of T1DM. Diabetic women typically have problems with their pregnancies; men with diabetes often experience fertility problems; adrenal insufficiency, kidney failure and retinopathy are common compli-

cations of T1DM; and so is impaired immunity resulting in unusual susceptibility to infectious diseases such as pneumonias. Anti-IR autoantibodies have been implicated in poor perinatal outcomes for fetuses born to diabetic women [40, 41] and autoantibodies against retinal pericytes are associated with increased retinal damage in diabetes [42,43], but it is not known whether these anti-retinal antibodies are targeted to IR. Indeed, no studies appear to have been done thus far on the possibility that anti-IR antibodies have pathological consequences in other tissues or organs compromised during the progression of T1DM. We propose that anti-IR autoimmunity may help to explain the range of dysfunctions that accompany the primary attack on the pancreas during T1DM.

The data further suggest that glucagon may be a target of autoimmunity in some patients with T1DM, as has been previously reported in some clinical cases [30, 31]. The novelty of the current study consists of demonstrating that the autoimmune response extends beyond antibodies to TCR sequences as well, and that these responses are not independent ones, but produce a highly integrated network of complementary interactions (Fig. 1).

### **Results in Light of Competing Theories of Autoimmunity**

The experimental results we report here have different possible meanings depending on the theory of autoimmunity within which they are interpreted. Many theories of autoimmunity have been proposed over the past sixty years, including T-cell bypass, T-cell dysfunction, epitope drift, hyperinflammation, etc., but most theories agree that microbial triggers are involved in the induction of most naturally occurring autoimmune diseases. The two theories based on microbial triggering that have garnered the most evidential support are the molecular mimicry theory (MMT) [44] and the antiidiotypic theory (AIT) [45].

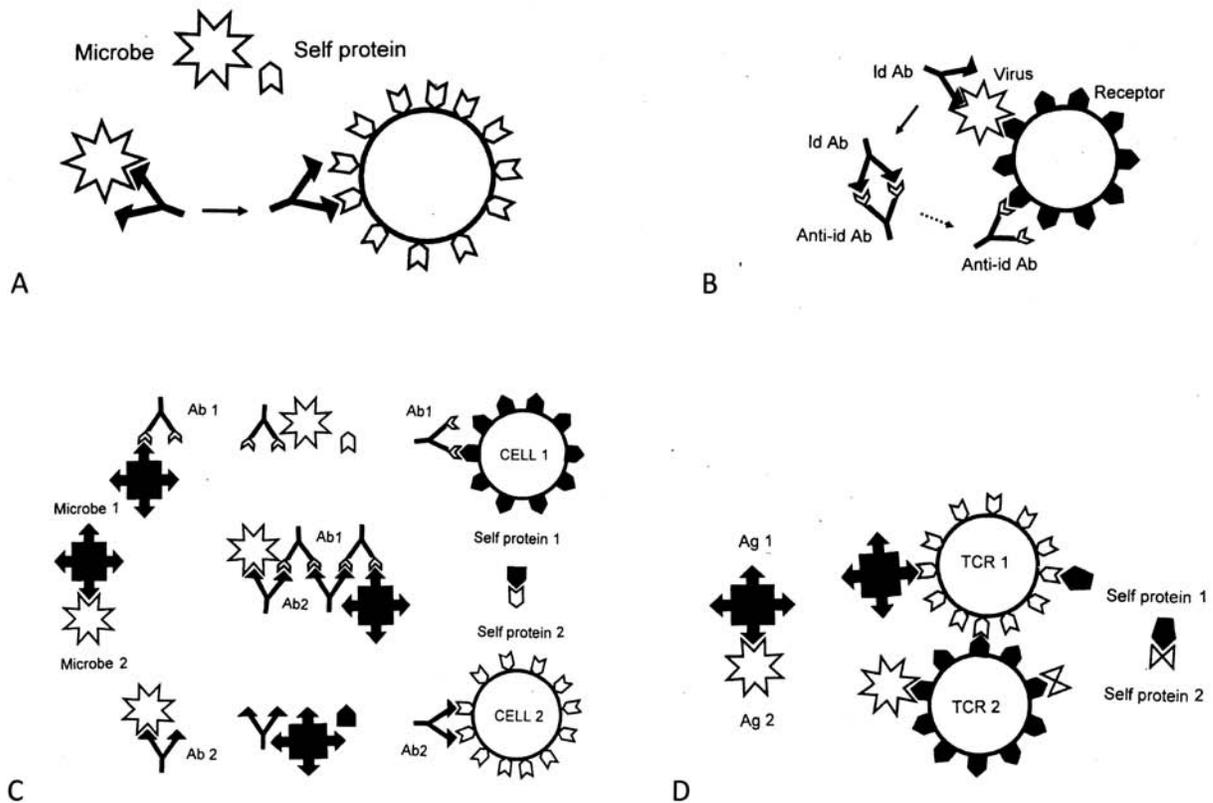
MMT proposes that microbes have antigens that mimic host proteins and that – for unknown reasons that may be explained by T-cell bypass, epitope drift, etc. – sometimes the body produces antibodies and/or T cells that cross react with these host proteins (Fig. 7A). According to MMT, the primary, idiotype immune response cross-reacting with the self mimic is all that is necessary to provoke autoimmunity if that response somehow escapes regulation. MMT does not, therefore, predict or account for the sets of complementary antibody or TCR responses characterized here.

In contrast to MMT, AIT proposes that since microbes often use cellular receptors to target host tissues or organs, antiidiotypic antibodies against viral antibodies will end up mimicking the microbe, thereby targeting its host tissue or organ (Fig. 7B). AIT is consistent with the complementarity between antibodies, between TCR and between antibodies and TCR characterized here, but assumes something that cannot be demonstrated here which is that this complementarity is due to the production of antiidiotypic responses from primary idiotypic responses to a single antigen. AIT does not explain why some individuals produce self-reactive antiidiotypic responses to any particular antigen when the vast majority of individuals do not. Moreover, according to AIT, the primary attack on insulin would have to be provoked by a primary immune response against something like glucagon

or IR, resulting secondarily in the production of anti-idiotype antibodies against insulin. Thus, while AIT can account for the appearance of complementary immune responses characterized here as idiotype-antiidiotype networks, AIT requires that some microbe exist that mimics the IR or glucagon, so that the anti-insulin response is secondary (antiidiotypic) rather than primary (idiotypic) as all current literature does.

While there is little doubt that molecular mimics play a role in the development of autoimmunity, and that what appear to be antiidiotype antibodies have been reported in T1DM [14-18, 34, 38], neither MMT nor AIT explains why autoimmunity results so extremely rarely from any particular insulin- or insulin-receptor mimicking microbial infection, nor does either theory explain how immune system dysregulation results to permit autoimmunity. Moreover, attempts over 30 years to elicit T1DM (or any other autoimmune disease) in animals using purified molecular mimics (without adjuvants) or antiidiotype antibody production have universally failed. We have therefore proposed a theory based on antigenic complementarity that accounts for the data supporting both MMT and AIT while also accounting for their deficiencies. The antigenic complementarity theory (ACT) of autoimmunity proposes that autoimmunity follows from normal immune responses against *concurrent* infections characterized by the unusual property of displaying molecularly complementary antigens, at least one of which mimics a host protein. Simultaneous exposure to complementary anti-

gens induces simultaneous, complementary immune responses expressed as antibodies (Fig. 7C), T cells or both (Fig. 7D). When both antibodies and TCR are activated, they will cross-react. These complementary immune responses *appear* to be idiotype-antiidiotype pairs, but actually arise simultaneously and independently of each other. Because the antigens are complementary, each immune response mimics one of the antigens. The mimicry between the antigens and the immune system responses to them results in the immune system losing the ability to distinguish self from nonself. Since at least one of the two antigens mimics a host protein as well, the loss of self-nonself discrimination permits an autoimmune response to proceed within the immune system itself as well as against a host tissue or organ. The constant presence of both host protein and, more importantly, complementary immune responses, ensures that the autoimmunity will be chronic because self-reinforcing. In sum, we propose that all autoimmune diseases are basically driven by the immune system attacking itself and only secondarily by the attack on a particular host tissue or organ. [25, 29, 46-49] ACT, like AIT, is also consistent with the complementary antibody and TCR interactions characterized here, but assumes that these complementary immune responses arise concurrently as independent responses to multiple, complementary antigens rather than as antiidiotype responses. Thus, ACT requires that there be both an insulin mimic and an IR or glucagon mimic to induce T1DM.



**Fig. (7).** A. A schematic drawing of the molecular mimicry theory (MMT). B. A schematic drawing of the antiidiotype theory of autoimmunity (AIT). C. A schematic drawing of the antigenic complementarity theory of autoimmunity (ACT) as applied to antibody production. D. ACT applied to TCR induction. ACT accounts for both molecular mimicry and the presence of antiidiotype antibodies in autoimmunity as well as complementary interactions among antibodies, TCR and BCR illustrated in Fig. (1), but not predicted by MMT or AIT.

### **MMT, AIT, ACT and Complementary Immune Responses: Causes or Down-Regulators of Autoimmunity?**

Several different, but not necessarily mutually exclusive, interpretations of the data presented above are possible depending on the theory from which one reasons. According to the Jerne network theory of immune regulation [50], anti-idiotypic antibodies are produced, in contrast to the AIT, as regulatory mechanisms for the idiotypic response [51, 52]. From this perspective, anti-TCR antibodies might also represent a regulatory mechanism invoked by the immune system to dampen the autoimmune response. Thus, Marcheloni [53] has reported that patients with rheumatoid arthritis and systemic lupus erythematosus have anti-TCR V beta autoantibodies that may limit the autoimmune reaction. The presence of complementary TCR in autoimmune patients with T1DM or other autoimmune diseases might similarly represent attempts by the immune system to control the autoimmune process. This possibility is enhanced by the fact that some of the TCR sequences listed in Table 2 mimic insulin, glucagon, and the insulin receptor. Perhaps the TCR that mimic insulin and insulin-receptor inhibit antibody that would otherwise attack hormonal and cellular targets. Such TCR may also target B-cells displaying insulin, glucagon, and insulin-receptor reactive B cell receptors, thereby down-regulating production of insulin, glucagon, and insulin-receptor reactive antibodies. Notably, however, interpreting antibodies against TCR as regulatory requires that the antibodies be anti-idiotypes, raising difficulties for both the MMT and AIT theories and opposing ACT.

### **Complementary Antibodies (and TCR) as the Cause of Autoimmunity**

An alternative interpretation of complementary TCR and antibodies in autoimmunity arises from the ACT. We [25, 29, 46-49] and others [54-57] have suggested that the simultaneous activation of two sets of complementary T-cell clones (or antibodies) by complementary antigens (as might occur in a mixed infection) would trigger each clone to recognize not only its respective antigen, but also its complementary T cell, as targets. Such T-cell targeting of other T cells would create an immunological civil war that could lead to the kind of immunological confusion typifying autoimmune diseases, including loss of the self-nonself distinction [25, 29, 46-49]. Labeling one set of TCR and antibodies as primary and the complementary set as being “anti-idiotypes” would then be misleading, since a key characteristic of anti-idiotypes is that they arise significantly after the primary immune response and as a direct response to it. It has been demonstrated by many investigators that anti-IR antibodies are not secondary to the induction of insulin antibodies, but are present from the outset of T1DM [14, 16, 18, 38].

Whatever interpretation is put on our findings, it appears that even beyond complementary antibodies and antibody-TCR complementarity, there is TCR-TCR complementarity in autoimmune diseases as well. Many of the diabetic TCR synthesized in this study recognized others more or less specifically. Indeed, the promiscuity of the TCR for each other was much greater than that observed for antibody-antibody binding, TCR-antibody binding, TCR-antigen binding or antibody-antigen binding. While these promiscuous TCR-

TCR results may be surprising, they should be placed in the context of the recent discovery that a single TCR sequence can recognize more than a million different peptide sequences [58]. Such TCR promiscuity is certainly not predicted from the Burnet clonal selection theory, but may play a significant role in immune system regulation (and dysregulation) if, for example, Marcheloni's explanation of anti-idiotypic networks holds up. From the perspective of MMT and AIT, such TCR promiscuity would seem to be counterproductive, almost ensuring that every possible microbe would find a promiscuous TCR mimic. From the perspective of ACT, TCR promiscuity would, unfortunately make TCR complementarity in response to any set of complementary antigens highly likely.

It is also notable that the TCR complementarity seems to be constrained within the set of diabetic TCR, since none of the diabetic TCR tested against TCR from HIV infected patients produced measurable binding. This finding suggests that TCR complementarity is induced as a specific part of the autoimmune process.

### **CONCLUSIONS**

In sum, the autoimmune response in diabetes is almost certainly more complex than is generally understood, and probably involves every possible set of complementary interactions that can occur between TCR, BCR and antibodies. The interactions appear, however, to be constrained to within this set of complementarities, rather than being randomly promiscuous. Targets include other TCR and antibodies and the insulin receptor in addition to the well-known targets of insulin and PTP-IA-2. Perversely, TCR not only target these proteins, but also mimic them. Thus, antigenic complementarity, such as that displayed between insulin and glucagon and between insulin and its receptor, also plays a fundamental role in the disease process by eliciting TCR that bind to other TCR as well. Understanding what triggers this set of complementary interactions, and how the set can be regulated, may provide new and important insights into the prevention and control of diabetes. Given Marcheloni's observation of antibody-TCR binding in other autoimmune diseases [53], it is likely that autoimmune diseases in general will turn out to display the antigen mimicry complicated by idiotypic-anti-idiotypic network of TCR-BCR-antibody interactions that we have characterized here.

Our results lead to a number of novel predictions that are unique to ACT, differentiating it from MMT and AIT. By extrapolation from TCR-TCR complementarity in T1DM, one would also expect B-cell receptors to be targets of TCR and of antibodies in autoimmunity, though this prediction has yet to be tested experimentally. Any theory of autoimmunity may need to account for the entire range of possible idiotypic-anti-idiotypic (or complementary antibody) interactions.

Another ACT-unique prediction follows from the observation that enteroviruses, especially coxsackie virus infections, are often associated with onset of T1DM [59-61] but that the timing of such infections is critical, sometimes leading to a protective effect [61]. Since MMT and AIT are based on single agent induction of autoimmunity, they cannot explain how timing can affect the outcome of infection.

ACT can explain the timing issue, since ACT requires a complementary coinfection to induce autoimmunity. An uncomplicated enteroviral infection would protect against diabetes.

ACT also makes other unique predictions. In T1DM cases associated with coxsackie infection, we predict that some of the TCR will be complementary to coxsackie antigens and some will also *mimic* coxsackie antigens. Further, the TCR that mimic coxsackie antigens will target the antigens of an infectious agent molecularly complementary to coxsackie virus. The nature of this complementary infectious agent will be identifiable from the TCR sequences that target coxsackie virus antigens because the complementary infectious agent will mimic these TCR sequences. Finally, a combination of these complementary antigens will induce pairs of complementary antibody and TCR responses that will cause T1DM as they arise. These predictions are unique to ACT and therefore provide a test of the alternative interpretations of the data provided above.

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#### CONFLICT OF INTERESTS

The authors have no conflicts of interest.

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