Brief Definitive Report

INSULIN B CHAIN FUNCTIONS AS AN EFFECTIVE COMPETITOR OF ANTIGEN PRESENTATION VIA PEPTIDE HOMOLOGIES PRESENT IN THE THYMUS

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Peptides derived from processing of complex protein antigens interact with products of the MHC to form the ligand recognized by T cells (1, 2). Recent crystallographic data support competitive binding studies showing that peptides restricted by the same MHC molecule bind to a limited number of sites on MHC (3–5). Self peptides that are nonimmunogenic include motifs that are predictive of MHC binding and can compete for Ia binding with similar immunogenic peptides, thereby modulating the response to the foreign peptide (6, 7). Therefore, self peptides may influence the response to foreign antigens in several ways: by competing for antigen presentation by binding to MHC sites; and through modifying the development of the T cell repertoire by clonal selection in the thymus of T cells bearing receptors reacting with self antigens as shown for I-E and Mls-reactive T cells (8, 9). The data presented here suggest that insulin B chain may function as an immunoregulatory peptide in man by both pathways: homology with the constant region of the TCR β chain results in a paucity of peripheral T cells recognizing insulin B chain; and B chain competes effectively at the level of the APC for presentation of immunogenic A chain determinants.

Materials and Methods

Antigens. Crystalline bovine insulin and *S*-sulfated human insulin B chain were the generous gift of Dr. Ron Chance (Lilly Research Laboratories, Indianapolis, IN). *S*-carboxymethylated bovine insulin B chain was synthesized in the laboratory of Dr. Dietrich Brandenberg (Deutsches Wollforschunginstitut, Aachen, FRG) and kindly provided by Dr. Alan Rosenthal (Merck Institute, Rahway, NJ).

Cells and Assay Conditions. Insulin-specific T cell clones 2/31, 2/37, and 3/50 were derived from a DR1, w6 diabetic and maintained by intermittent stimulation with insulin, IL2, and irradiated autologous mononuclear cells (MNC) as described (10). APC used were irradiated autologous MNC or EBV-transformed B cells (EBV B cells). Proliferation assays were performed as described (10) in triplicate in U-bottomed plates; each well contained 2×10^4 T cells and 10^5 irradiated APC in 0.2 ml. Cultures were incubated for 72 h and received 1 μCi methyl-[^{3}H]thymidine during the final 18 h of incubation. Data are the mean cpm ± SD of triplicate wells. For antigen pulsing experiments, EBV B cells were incubated overnight with insulin and/or inhibitor (25 μM each) at a final density of 1.5×10^6/ml in 24-well flat-bottomed plates. Washed cells were fixed in paraformaldehyde for 1 min as previously detailed (11).

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Results and Discussion

Insulin B Chain Competes with Immunogenic A Chain Determinants. Human T cell clones specific for insulin were derived from an insulin-treated diabetic; these T cells require processing of insulin and recognize determinants on the A chain of bovine insulin in association with HLA-DR1 (10, 11). In contrast to guinea pig T cells (12), no human T cell clones were found that recognized insulin B chain. We therefore examined the effects of insulin B chain on the response of three beef insulin-specific T cell clones with differing fine specificities (11). The response of clone 3/50 to insulin was inhibited in a dose-dependent fashion by isolated B chain (Fig. 1 *A*). Inhibition was detected at 1 μM B chain, and the response was completely inhibited by 30 μM B chain. Inhibition was found with either autologous MNC or EBV B cells as APC. Both human and bovine insulin B chain, differing by one amino acid (B30), were inhibitory, irrespective of the modification of the cysteines (*S*-carboxymethylated or *S*-sulfated). Similar results were found for clones 2/31 and 2/37 (Fig. 1 *B* and data not shown).

To ascertain if the B chain was acting on the TCR complex, we examined the response of clone 2/37 (Fig. 1 *B*). This clone has dual specificity for the A loop of beef insulin in association with HLA-DR1 and is also alloreactive to a determinant expressed on cells bearing HLA-DR3DQw2 molecules in the absence of insulin (10). As shown, B chain inhibited the insulin response of this clone while the alloreactivity was not affected. These experiments excluded nonspecific toxicity of B chain and show that inhibition was probably not due to direct interaction with the TCR complex. In additional experiments, T cells maintained in the presence of B chain were fully responsive to antigen excluding tolerization as a mechanism for inhibition (data not shown).

To further examine the ability of B chain to compete with A chain determinants for presentation to T cells, the following experiments were carried out. APC were pulsed with insulin alone, insulin plus B chain, or B chain alone and allowed to process antigen prior to fixation. T cell response to insulin-pulsed, fixed APC was inhibited by the addition of B chain to the cultures (Fig. 2 A). When APC were

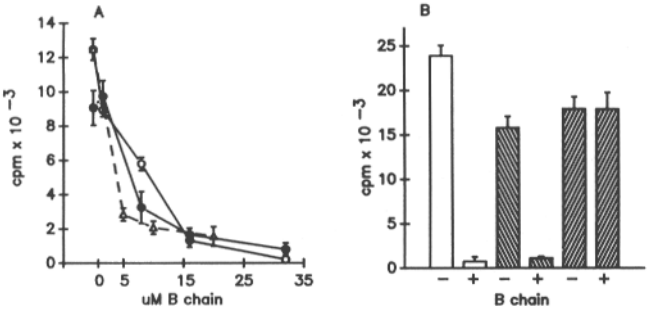

FIGURE 1. Inhibition of T cell response to beef insulin by insulin B chain. (*A*) Clone 3/50 was cultured with 5 μM beef insulin and APC (○, △, autologous mononuclear cells; ●, EBV B cells) in the absence or presence of the indicated concentrations of B chain (—, *S*-sulfated human insulin B chain; - -, *S*-carboxymethylated bovine insulin B chain). (*B*) Clone 2/37 was cultured with autologous mononuclear cells (□) or DR1 EBV B cells (▧) plus 10 μM beef insulin, or with DR3DQw2 EBV B cells (▨) without insulin in the absence or presence of 30 μM *S*-sulfated B chain. Controls: (*A*) clone 3/50 plus mononuclear cells alone, 201 ± 56; clone 3/50 plus EBV B cells alone, 1,702 ± 109; EBV B cells alone, 989 ± 104. (B) Clone 2/37 plus mononuclear cells, 386 ± 198; 2/37 plus DR1 EBV B cells, 1,287 ± 212; DR1 EBV B cells alone 1,300 ± 96; DR3DQw2 EBV B cells alone, 1,064 ± 165.

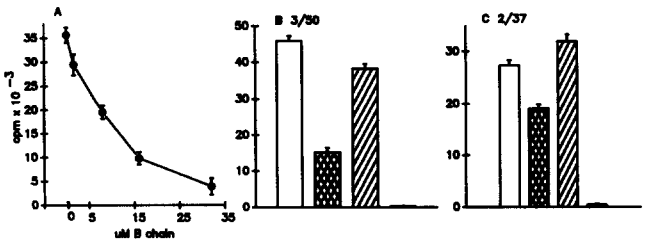

FIGURE 2. B chain competes with insulin for antigen presentation. (*A*) EBV B cells were pulsed with beef insulin and fixed with paraformaldehyde before use as stimulators for 3/50 in the absence or presence of the indicated concentration of human insulin B chain. (*B*) 3/50 T cells were cultured with irradiated EBV B cells that had been pulsed with beef insulin alone (25 μM) (□), simultaneously with beef insulin plus B chain (25 μM each) (▩), pulsed separately with insulin (25 μM) and B chain (25 μM) and mixed (▨), or B chain alone (25 μM) ■), before fixation with paraformaldehyde. (*C*) Same as *B*, except 2/37 T cell responders. Control responses (*A*, *B*) 3/50 plus nonpulsed EBV B cells, 1,072 ± 109; EBV B cells alone, 1,100 ± 202: (*C*) 2/37 plus nonpulsed EBV B cells, 1,325 ± 226; EBV B cells alone, 974 ± 276.

pulsed simultaneously with insulin and B chain prior to fixation, inhibition was also observed (Fig. 2, *B* and *C*). However, if the APC were pulsed separately with insulin or B chain and then mixed, the T cell response was not inhibited (Fig. 2, *B*, *C*). Thus, the B chain competes with other insulin epitopes at the surface of APCs.

Sequence Homology of Insulin B Chain and TCR β Chain Constant Region. The amino acid sequence of insulin B chain includes several motifs that are frequently predictive of T cell epitopes (13) including several appropriately spaced hydrophobic residues that are recognized by other DR1-restricted human T cells (14). The A chain of insulin includes only one such motif proximal to the A loop which stimulates the clones described here, but none of our clones recognize B chain. When we searched Genebank for proteins with homology to insulin B chain, two clusters of amino acid sequence identity with human and murine TCR β chain constant regions were found (Fig. 3). Therefore several B chain peptides were tested for inhibition of antiinsulin responses (Table I). These peptides include subunits spanning the entire B chain, but none of them individually or in mixtures were inhibitory. Peptides spanning residues 16–26 of the B chain and corresponding peptides of the TCR β chain have thus far been insoluble in any diluent that is nontoxic to the cell cultures. We have been unable to test directly the hypothesis that both homology clusters must be present on the same peptide for inhibition.

These experiments demonstrate that a nonimmunogenic portion of the insulin molecule inhibits the response to immunogenic epitopes of the same molecule. Together with data on DR1-restricted recognition by other T cell clones (14), the most direct conclusion is that insulin B chain competes with processed A chain epitopes for MHC binding. This conclusion is supported by recent studies that show that B chain binds murine class II MHC molecules from responder and nonresponder haplotypes (15). An alternative mechanism consistent with the data is binding of B chain by MHC leading to inhibition of the interaction between class II molecules

Insulin B chain	Y L V C G E R G F F Y
TcR $c\beta_2$ human	T L V C L A T G F F P
Murine $c\beta$	T L V C L A R G F F P

FIGURE 3. Sequence homology of insulin B chain (B16-26) and the first exon of TCR β chain constant regions (140–150).

TABLE I

Intact B Chain Is Required to Inhibit T Cell Response to Insulin

B chain peptide	Exp. 1	Exp. 2
None	11,341	5,671
B chain (1-30)	4,852	917
B (1-8)	11,579	NT
B (1-16)	11,572	6,703
B (1-23)	11,414	6,041
B (17-23)	10,114	NT
B (24-30)	11,145	NT
B (1-23) + B (24-30)	NT	5,100
B (1-16) + B (24-30)	NT	6,280
B (17-23) + B (24-30)	NT	6,712

Clone 3/50 was cultured with irradiated DR1 EBV B cells plus 10 μM beef insulin (Exp. 1) or mononuclear cells plus 5 μM beef insulin (Exp. 2) in the absence (none) or presence of B chain [B (1-30)] or peptides (20 μM final concentration). Results are Δ cpm for triplicate assays. Controls: Exp. 1, clone plus DR1 EBV B cells alone 5,733 $\pm$ 980; Exp. 2, clone plus mononuclear cells alone 650 $\pm$ 132.

and CD4. In either case, the effects of the B chain provide a mechanism by which self peptides may modify the response to foreign antigens.

The homology with the constant region of the TCR β chain indicates that B chain-related epitopes are also present in the thymus during development of the T cell repertoire. This homology is found in both human and murine T cell receptor β chains. Analysis of the specificity of murine T cell hybridomas has demonstrated the dominance of A chain epitopes in the response to beef and pork insulin (16, 17). Although some of these T cells recognize determinants that include the proximal B chain disulfide linked to A chains (17), no murine clones responsive to isolated B chains have been reported. B chain responsive T cells are found in strain 13 guinea pigs (12); these T cells recognize a determinant (B5-16) proximal to the sequences homologous to the TCR. In man, polyclonal T cells were reported to react to isolated B chains and somewhat to A(16-21)/B(10-25), but poorly or not at all to A(20-21)/B(17-25) (18). Intrathymic homology may be one means of establishing T cell tolerance to self molecules that are not present in the thymus and provide an additional mechanism by which self peptides modify responses to foreign antigens. If the proposed use of peptides as immunotherapy of autoimmune disease is to be successful, these experiments suggest that the most effective peptides should include motifs that predict interaction with MHC and are present in the thymus during T cell development.

Summary

The B chain of mammalian insulins contains appropriately spaced amino acids that predict recognition by T cells. However, all T cell clones from an HLA-DR1, Dw6 diabetic donor recognize epitopes associated with the A chain, and the B chain was found to inhibit these responses. Effective intramolecular competition at the level of the APC, not a direct effect on the T cell, is responsible for the inhibition. Insulin B chain contains two clusters of amino acid homology with the

TCR β chain and B chain peptides lacking these clusters do not compete for antigen presentation. A hole in the repertoire for T cells that recognize this portion of the insulin molecule may arise in the thymus by deletion of T cells that recognize similar peptides.

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