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Bovine and Human Insulin Activate CD8+ -Autoreactive CTL Expressing Both Type 1 and Type 2 Cytokines in C57BL/6 Mice

Hakling Ma,∗ Yong Ke,† Qingqin Li,‡ and Judith A. Kapp§

CD8+ T cells down-regulate a variety of immune responses. For example, porcine and human insulin do not stimulate Abs in C57BL/6 mice because CD8+ T cells inhibit CD4+ helper T cells. By contrast, bovine insulin induces Ab in C57BL/6 mice, and removal of CD8+ T cells does not alter this response. This raises the question of whether porcine, but not bovine, insulin activates CD8+ T cells or whether both insulins activate CD8+ T cells but CD4+ helper T cells are differentially inhibited by them. In this study, we show that insulin-specific CD8+ CTL can be cultured from C57BL/6 mice primed with either bovine or human insulin in CFA. Thus, exogenous Ags, besides OVA, induce CD8+ CTL when administered in an adjuvant, suggesting this is a typical response. These CTL are H-2Kb restricted and produce IL-5, IL-10, INF-γ, and small amounts of IL-4, which is distinct from IFN-γ and TNF-α that are typically secreted by virus-specific CTL. Moreover, the CTL primed with either bovine or human insulin recognize an A-chain peptide that is identical to the mouse insulin sequence. That foreign proteins, which are closely related to self-proteins, activated autoreactive, CD8+ T cells in vivo is a novel finding. It raises the possibility that self-reactive CTL may be activated by cross-reacting Ags and once activated they might participate in autoimmunity. These results also suggest that down-regulation of insulin-specific responses by autoreactive CD8+ T cells is most likely due to the differential sensitivity of bovine and human insulin-specific CD4+ T cells. The Journal of Immunology, 2000, 164: 86–92.

Many investigators have reported that exogenous Ag activate CD8+ T cells that specifically inhibit immune responses upon adoptive transfer to naive recipients. For example, porcine insulin (PINS), which fails to stimulate Ab responses in C57BL/6 mice, does so if radioresistant CD8+ T cells are removed (1, 2). Such suppressive activity has been puzzling because CD8+ T cells recognize predominantly endogenous Ag that is processed via the MHC class I pathway. In addition, most cells do not take up or present exogenous proteins, such as OVA, to class I MHC-restricted T cells (3). However, phagocytic cells are a very important exception to this rule since they process OVA for presentation to both MHC class II- and class I-restricted T cells (4–7).

Injection of native OVA i.v. (8), i.p. (9), or s.c. (4, 10) does not generally prime CTL. However, spleen cells pulsed with OVA prime OVA-specific CTL (OVA-CTL) upon transfer to syngenic mice (3). Spleen cells pulsed with OVA also prime host-resticted, OVA-CTL in mice expressing a different MHC haplotype (8). Such cross-priming suggests that foreign cells, cell debris, or membrane fragments, are taken up by APC that reprocess the Ag and present it in the context of self-class I MHC molecules (11). It is now also clear that administration of exogenous proteins with adjuvants activates CTL (reviewed in Ref. 12). For example, we have shown that OVA emulsified in CFA primes OVA-specific CD8+ CTL (10). Both oil and mycobacteria were required for activating the CTL and priming was not due to contaminating peptides. Priming of CTL by OVA in CFA requires phagocytic cells but not CD4+ T cells. Presumably, CD4+ T cells are not required for priming because the mycobacteria directly activate APC (13).

OVA-CTL can act as suppressor T cells in that they inhibit OVA-specific CD4+ T cell responses in vitro (14) and generation of Ab and priming CD4+ in vivo (10). The studies of Rock et al. (14) also demonstrated that phagocytic cells serve as targets for CTL. Our studies showed that B cells also serve as targets for OVA-CTL provided that the Ag is taken up by surface Ig-mediated endocytosis (15). Thus, one potential mechanism by which CTL can specifically inhibit responses of CD4+ T cells is by the lysis of APC that process the Ag into both MHC class I and class II pathways.

Previously, we reported that injection of PINS in CFA stimulates CD8+ T cells that prevent the development of an Ab response in nonresponder C57BL/6 (B6) mice (1, 2). In addition, CD4+ helper T cells have been cloned from nonresponder mice primed with PINS or human insulin (HINS) (16). Insulin-specific (INS) CD4+ T cells also have been cloned from BALB/c mice rendered nonresponsive to HINS by the pancreatic expression of the HINS transgene (17). In contrast to PINS, bovine insulin (BINS) induces an Ab response in B6 mice, and removal of CD8+ T cells did not alter the helper activity of the remaining CD4+ T cells (1). These observations suggested that PINS and HINS, but not BINS, may activate INS CD8+ T cells in B6 mice. Alternatively, all forms of
insulin might activate CD8+ T cells but helper T cells specific for HINS and PINS might be more sensitive to suppression by CD8+ T cells than helper T cells specific for BINS. Studies in this report were designed to test these possibilities using insulin-transfected EL4 cells as stimulators and targets for CD8+ CTL.

Materials and Methods
Mice
Female 8- to 12-wk-old B6 (H-2b) mice were purchased from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). All procedures on animals were conducted according to the principles outlined in the guidelines of the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources (National Research Council, Washington, DC).

Reagents
Recombinant HINS was a gift from Eli Lilly (Indianapolis, IN) and purified BINS was purchased from Sigma (St. Louis, MO). CFA containing Mycobacterium tuberculosis, strain H37Ra, was obtained from Difco (Detroit, MI). Peptides used in this paper (Table I) were kindly provided by Dr. James M. Sheil (West Virginia University, Morgantown, WV) or Dr. Peter E. Jensen and Dr. Brian Evavold (Emory University, Atlanta, GA) or synthesized by the Emory University Microchemical Facilities. Monoclonal IgG2a Abs specific for H-2Ld/H-2D b (28-14-8) (18) and H-2K b (AF6-88.5) (19) were purchased from Pharmingen (San Diego, CA).

Tumor cells
E.G7-OVA, generated by transfection of EL4 with the OVA cDNA (3), was provided by Dr. Michael J. Bevan (University of Washington, Seattle, WA). EL4-INS, P815-INS, and P815-INS were generated by transfection of EL4 (H-2 b ), M12.4.1 (H-2 d ), and P815 (H-2 d ) tumor cell lines by electroporation at a voltage of 300 V and capacitance of 400 μF. Selection in G418 at 1.0 mg/ml was begun 24 h after electroporation. The neomycin-resistant cells were cloned and they are referred to as EL4-INS, M12-INS, and P815-INS.

Northern blot analysis
Total RNA was prepared using the Ultraspec RNA isolation system (Biotex, Houston, TX). Purified total RNA (15 μg) was electrophoresed in a 1.2% agarose-formaldehyde denaturing gel, transferred to nitrocellulose membranes (Boehringer Mannheim, Indianapolis, IN), and then cross-linked to the nitrocellulose with UV Stratalinker (Stratagene, La Jolla, CA). The membranes were hybridized at 65°C in the rapid hybridization buffer (Amerham, Arlington Heights, IL) for 15 min and then hybridized at 65°C with a 32P-labeled HINS cDNA (Ncol-HindIII fragment) probe for 2 h (22). The blots were washed in 2× standard saline citrate phosphate/EDTA (SSPE)/0.1% SDS, 1× SSPE/0.1% SDS, and 0.7× SSPE/0.1% SDS for 15 min each and then exposed to x-ray films (DuPont Pharmaceuticals, Boston, MA) at ~70°C to reveal hybridization signals.

Establishment and maintenance of T cell lines and clones
CTL were generated by immunizing B6 mice s.c. in the footpad with 100 μg of BINS, HINS, or OVA in CFA. After 10 days, 3×106 splenocytes were cultured for 1 wk with 3×106 irradiated (20,000 rad) stimulator cells (EL4-INS or E.G7-OVA). Thereafter, T cells were restimulated weekly with irradiated EL4-INS or E.G7-OVA, irradiated syngeneic splenocytes, and 20 μ/ml recombinant human IL-2 (kindly provided by Dr. Maurice Gately, Hoffmann-La Roche, Nutley, NJ). After several passages, T cells were cloned by limiting dilution.

51Cr release assay
Cytotoxicity against targets was quantified in a 4-h 51Cr release assay (10). Briefly, syngenic (H-2b) targets (E.G7-OVA, EL4-INS, or E.G7-OVA) or allogenic (H-2d) M12-INS target cells were labeled with Na2Cr51O4 (DuPont Pharmaceuticals) at 37°C for 1 h. After washing, 51Cr-labeled target cells were incubated with INS-CTL or OVA-CTL at different E:T ratios in 96-well round bottom plates. After a 4-h incubation at 37°C, supernatants were collected and radioactivity was detected in a gamma counter (Wallac, Turku, Finland). Percentage of specific lysis was calculated as 100 × [(release by CTL – spontaneous release)/(maximal release – spontaneous release)]. Maximal release was determined by addition of 1% Triton X-100 (EM Science, Gibbstown, NJ). Spontaneous release in the absence of CTL was generally <15% of maximal release.

Cytokine ELISA
Effector T cells (106) were incubated with target cells (5×105) for 24 h, and supernatants were collected and assayed for lymphokines using paired mAbs specific for IL-2, IL-4, IL-5, IL-10, IFN-γ, or TNF-α (Pharmingen). Biotinylated Ab were added and detected with avidin-peroxidase (Vector Laboratories, Burlington, CA) plus 2,2-azino-di-[3-ethyl-benzthiazoline sulfonate] (substrate containing H2O2 (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The colorimetric reaction was read at 450 nm using an automatic microplate reader (Molecular Devices, Menlo Park, CA). The
concentrations of the cytokines were calculated from the standard curve of the appropriate recombinant cytokines.

**Results**

**Generation of HINS-transfected cell lines**

A genomic clone of the HINS gene was subcloned into an expression vector containing the human β actin promoter and the SV2-neo gene (23) that was used to produce E.G7-OVA (3). The resulting plasmid, pHβApr-1-neo-HINS (Fig. 1A), was transfected into EL4, M12.4.1, or P815 by electroporation. Transfectants were selected in G418, cloned, and tested for the expression of the insulin gene by Northern blot analysis. The transfected cells (EL4-INS, M12.4.1-INS, and P815-INS) expressed the 0.55-kb mRNA (Fig. 1B, lanes 2, 4, and 6) that was expected for insulin (22), whereas the untransfected parental lines did not (Fig. 1B, lanes 1, 3, and 5).

**Generation and characterization of INS-CTL**

To generate INS-CTL, B6 mice were primed with 100 μg of BINS or HINS in CFA in the hind footpad. After 10 days, splenocytes were cultured with EL4-INS as described in Materials and Methods. Spleen cells from insulin-primed mice usually required three to four cycles of restimulation before lytic activity was detected (data not shown). Both BINS-CTL (Fig. 2A) and HINS-CTL (Fig. 2B) lines lysed EL4-INS targets in a dose-dependent manner. INS-CTL did not lyse EL4 cells transfected with the OVA gene (EG7-OVA), demonstrating that they are INS-CTL. Target cells expressing insulin but the wrong MHC class I haplotype, such as M12-INS (Fig. 2A and B) or P815-INS (data not shown), were not lysed by INS-CTL, suggesting that the CTL are MHC restricted. In addition, MHC restriction of the INS-CTL maps to class I because EL4 and transfectants of EL4 do not express MHC class II Ags. In contrast to the INS-CTL, OVA-CTL induced by immunization with OVA in CFA (10) recognized E.G7-OVA but not EL4-INS or M12-INS (Fig. 2C), verifying the specificity of the target cells and CTL. To date, B6 mice primed with insulin in CFA have reproducibly generated INS-CTL in 12 separate experiments. Priming is required since cytolytic activity is not stimulated by EL4-INS in cultures of spleen cells from naive mice, as previously observed for OVA-CTL (3, 10).

The cell surface phenotype was determined for BINS-CTL and HINS-CTL by flow cytometry. Both expressed CD8, CD3, and αβ TCR but not CD4 (data not shown). The cytokines produced by BINS-CTL and HINS-CTL were determined by incubating the CTL alone or with irradiated E.G7-OVA or EL4-INS cells for 24 h. The supernatants were then examined by ELISA for the presence of various cytokines (Fig. 3). Neither BINS-CTL nor HINS-CTL produced detectable amounts of cytokines in the absence of stimulators. Both CTL produced IL-5, IL-10, IFN-γ, and some IL-4 (about 100 pg/ml) but no detectable IL-2 or TNF-α in

**FIGURE 1.** Transfection of tumor cells. A, Restriction map of pHβApr-1-neo-HINS used for transfecting EL4 (H-2b), M12.4.1 (H-2k), and P815 (H-2d). Restriction sites include E, EcoRI; H, HindIII; N, NcoI (eliminated in cloning); and S, SalI. B, Northern blot analysis of the HINS gene expression in transfected and untransfected tumor cells using 32P-labeled HINS cDNA (NcoI-HindIII fragment) as probe.

**FIGURE 2.** Generation of INS-CTL. Spleen cells from B6 mice primed with 100 μg of BINS (A) or HINS (B) in CFA were restimulated in vitro four times. The CTL cell lines were then analyzed for specific lytic activity using 51Cr-labeled E.G7-OVA, EL4-INS, and M12-INS targets in a standard 4-h Cr51 release assay. OVA-CTL from B6 mice primed with OVA in CFA were similarly tested for lytic activity (C).
the presence of EL4-INS cells. However, TNF-α is produced by OVA-CTL (24) and IL-2 is produced by several INS CD4⁺ clones (25). BINS-CTL and HINS-CTL secreted low but detectable levels of the same cytokines when stimulated with E.G7-OVA, which probably represents a low level of cross-reactivity with the OVA peptide.

Secretion of IFN-γ, IL-4, IL-5, and IL-10 suggested that the INS-CTL bulk lines consisted of a mixture of T cells that produce either type 1 or type 2 cytokines or a single population that produces both cytokines. To address this question, BINS-CTL were cloned by limiting dilution. Seven of eight clones were lytic as illustrated by B2.2 (Fig. 4A). These clones produced IL-5, IL-10, IFN-γ, and low levels of IL-4 (about 100 pg/ml) (Fig. 4B) as did the bulk line. One of the eight clones (B2.8) lacked lytic activity for EL4-INS targets (Fig. 4C) but produced a cytokine profile similar to that of the bulk line and clone B2.2 (Fig. 4D). Three of three clones derived from the HINS-CTL bulk culture displayed lytic and cytokine profiles similar to those of the bulk line (data not shown). These data suggest that both BINS-CTL and HINS-CTL can be classified as Tc0 cells.

MHC restriction and peptide specificity of INS-CTL

To identify the specific restriction element for BINS-CTL, mAbs specific for H-2Db (28-14-8) or H-2Kb (Af6-88.5) were tested for the ability to inhibit activation of CTL by EL4-INS (Fig. 5). Anti-H-2Kb significantly inhibited cytokine production by BINS-CTL, whereas anti-H-2Db mAb inhibited little, if at all. Anti-H-2Kb significantly inhibited cytokine production by HINS-CTL (Fig. 5). Anti-H-2Db partially suppressed the responses of HINS-CTL, suggesting a small population might be Db restricted. However, BINS-CTL and HINS-CTL recognize insulin presented primarily by the H-2Kb molecule.

To map the epitope recognized by INS-CTL, EL4 were initially pulsed with different insulin peptides. However, EL4 cells alone stimulated significant amounts of cytokine production by INS-CTL (data not shown). Reactivity to EL4 could be due to the cross-reactivity of endogenous peptides that are bound to MHC class I with the insulin peptides, as Forquet et al. (26) encountered the same problem with cells transfected with hen egg lysozyme.
This interpretation was supported by removing endogenous peptides from class I molecules on EL4 cells with an acid-stripping method (27). Acid-treated EL4 cells no longer stimulated INS-CTL and acid-stripped EL4 cells pulsed with BINS fragments activated INS-CTL efficiently (data not shown).

To circumvent the need to acid strip EL4 cells for peptide-mapping studies, RMA-S cells (21) were used as APC. RMA-S cells lack the Tap-1 gene product that is required to transport cytosolic peptides into the endoplasmic reticulum, where they bind to class I molecules. Consequently, the empty class I molecules that are transported to the surface of RMA-S cells are unstable. The surface class I molecules can be stabilized by binding exogenous peptides, and thus RMA-S pulsed with peptides are effective stimulator cells (28).

RMA-S cells pulsed with 10 \( \mu \)M chymotryptic fragments or synthetic peptides of insulin (Table I) were used as stimulator cells for BINS-CTL and HINS-CTL. Supernatant fluids were collected after 24 h and assayed for IL-5 production as a measure of T cell activation (Fig. 6). As expected, RMA-S cells pulsed with intact BINS or PINS did not activate BINS-CTL or HINS-CTL. RMA-S cells pulsed with chymotryptic peptides of BINS activated both CTL lines. In addition, chymotryptic peptides from the BINS A-chain, but not the B-chain, activated both CTL lines. To identify the epitope of the A-chain more precisely, synthetic peptides of PINS and BINS A-chain were tested for activation of INS-CTL. Neither the PINS nor BINS A-chain\(_{1-14}\) activated either of the INS-CTL. However, a low level of activation was stimulated by A-chain\(_{8-21}\), and the maximum response was induced by A-chain\(_{12-21}\).

Truncated A-chain\(_{12-21}\) peptides were used to identify the minimal epitope recognized by BINS-CTL and HINS-CTL lines. The A-chain\(_{12-21}\) peptide stimulated maximum responses by both CTL lines as measured by IL-5 production (Fig. 7). Removal of amino acid residue Ser\(_{12}\) from the N terminus dramatically reduced the capacity to stimulate both INS-CTL. Omission of Asn\(_{21}\) from the C terminus inhibited IL-5 production by BINS-CTL, although some residual activity was detected by production of IL-10 (data not shown). Thus, A-chain\(_{12-21}\) is the most effective peptide for activation of BINS-CTL. Seven of the eight BINS-CTL clones that have been tested were also activated by A-chain\(_{12-21}\). The other clone did not recognize any of the synthetic fragments tested (data not shown), suggesting that the epitope might span the two peptides. Ser\(_{12}\) was also critical for recognition by HINS-CTL but the requirement for residues 20 and 21 was not as strict as in BINS-CTL. HINS-CTL clones all recognized the same insulin A-chain fragments as the bulk line (data not shown).
Discussion

These experiments demonstrate that INS CD8+ CTL are activated by priming B6 mice with BINS or HINS in CFA. Lytic activity was not detectable for several weeks after culture initiation with EL4-INS stimulators. By contrast, priming B6 mice with OVA in CFA stimulates detectable cytolytic activity after 1–2 wk of culture with E.G7-OVA. These differences may reflect a lower frequency of INS-CTL precursors or the recruitment of CTL with low avidity. Nevertheless, both insulin and OVA activate CTL, suggesting that CTL may be a normal consequence of priming with exogenous proteins in the presence of adjuvants.

Most of the INS clones were cytolytic but noncytolytic clones have been obtained occasionally. Both lytic and nonlytic INS clones produced IFN-γ, IL-5, IL-10, and low amounts of IL-4. This pattern of cytokines differs from CTL primed with OVA in CFA, which produce IFN-γ and TNF-α exclusively (29). The cytokine milieu clearly plays a role in polarizing CD8+ T cells in ways that are similar to, yet distinct from, those regulating CD4+ T cells (30). However, our results suggest that the functional phenotypes of CD8+ T cells may be as complex as CD4+ T cells, and the antigenic specificity and avidity of the responding CD8+ T cells may play a significant role in the pattern of cytokines they produce.

CTL stimulated by BINS and HINS recognize the A-chain12–21 peptide of insulin presented primarily by the H-2Kb molecule. However, HINS-CTL recognize A-chain12–19 more strongly than do BINS-CTL. The amino acid sequence of A-chain12–21 Peptide is identical in BINS and HINS. INS-CTL have also been generated by incubating naive H-2b splenocytes with chymotryptic digests of BINS in vitro which gave rise to CTL that recognized the B-chain7–15 peptide (31). However, EL4-INS cells are capable of activating peptide-induced INS-CTL. J. M. Sheil, personal communication. Differences in epitope specificity of INS-CTL described here and that reported by Sheil et al. (31) may be attributed to different methods of priming. Previously, Carbone et al. (32) demonstrated that the OVA-CTL induced by incubation of naive T cells with OVA peptides recognized several epitopes that were distinct from OVA-CTL primed with E.G7-OVA cells in vivo.

Although CTL generated with intact BINS or HINS in CFA or insulin fragments recognize distinct epitopes on different chains of insulin, all are specific for highly conserved sequences of insulin. Mouse insulin sequences (Table I) demonstrating that all of these insulin fragments recognize distinct epitopes on different chains of insulin, all are specific for highly conserved sequences of insulin.

The idea that CD8+ T cells with high avidity for insulin might be deleted in the thymus is supported by the observations that genes linked to the insulin promoter are expressed in the thymus (34, 35). Insulin and other pancreatic genes, such as elastase, are also expressed in the thymus where they have been shown to induce tolerance in CD8+ T cells (36, 37). Transplantation of the thymus under the kidney capsule of nude mice confers complete tolerance in maturing CD4+ T cells and significant, but variable, levels of tolerance in CD8+ T cells (38). We hypothesize that the high-avidity, self-insulin-specific CD8+ T cells may be tolerated in the thymus, whereas low avidity T cells specific for A-chain12–21 and B-chain7–15 peptides are not.

In nonobese diabetic mice, CD8+ CTL collaborate with CD4+ Th1 cells to induce diabetes (reviewed in Ref. 39). The specificity of these CD8+ T cells has been unknown, but a clone isolated from the pancreas of a nonobese diabetic mouse has recently been reported to recognize insulin B-chain12–23 (40). This raises the question of whether the CD8+ CTL that recognize autologous insulin, which are described in this communication, could cause autimmune diabetes. However, mice immunized with INS in CFA never develop gross evidence of diabetes, such as increased urinary output or increased blood glucose levels (our unpublished observations). Moreover, primed mice that were boosted several times with INS failed to develop evidence of pancreatic infiltration beyond the occasional interstitial infiltration of mononuclear cells seen in control mice primed with CFA alone (41). The failure of the INS-CTL to induce diabetes cannot be attributed to the lack of CD4+ helper T cells because they are activated by both BINS and HINS in CFA (1, 16, 17). It is not clear whether the failure to develop diabetes in B6 mice is due to a precursor frequency that is below the threshold to produce disease, expression of TCR with low avidity, the lack of other essential signals required to target the CTL to the pancreas, the cytokine profile of the INS-CTL, or other factors.

Previously, we reported that injection of PINS in CFA stimulated CD8+ T cells that prevent the development of an Ab response in nonresponder B6 mice (1, 2). Removal of the CD8+ T cells revealed that radioresistant, CD4+ helper T cells had been primed by PINS. By contrast, BINS induces an Ab response in B6 mice and removal of CD8+ T cells did not alter the helper activity of the remaining CD4+ T cells (1). From these observations, we suggested that PINS (and HINS), but not BINS, primed CD8+ T cells in B6 mice. The data presented here suggest that the suppression previously attributed to PINS- or HINS-induced CD8+ T cells may be attributed to the lytic activity or the cytokine production by INS CD8+ T cells. However, contrary to our original prediction, both HINS and BINS stimulated CD8+ CTL. Thus, differences in Ab responses to BINS and HINS in B6 mice cannot be attributed to differences in the ability to prime CD8+ CTL. However, HINS-CTL recognized a broader spectrum of peptides than did BINS-CTL and exhibited low level responses to Dα as well as Kβ. Thus, the broader reactivity of HINS-CTL could contribute to greater inhibition of responses to HINS than BINS. Alternatively, CD4+ T cells primed with HINS or BINS might differ in their sensitivity to inhibition by CD8+ T cells. Studies are currently underway to test these possibilities.

The data presented here demonstrate that self-reactive CD8+ T cells can be activated by exogenous, foreign proteins. CD8+ T cells have been previously shown to down-regulate Ab responses to certain of these cross-reactive, exogenous Ags (1, 2). Thus, we suggest that autoreactive CD8+ T cells may play an important role in actively shaping the functional repertoire of responses to exogenous Ags. However, direct evidence that the CD8+ CTL described in this manuscript are relevant to the previously described suppressive effects awaits future in vivo experiments.

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