REGULATORY MECHANISMS IN IMMUNE RESPONSES TO HETEROLOGOUS INSULINS

II. Suppressor T Cell Activation Associated with Nonresponsiveness in H-2b Mice

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Murine antibody responses to heterologous insulins are qualitatively controlled by MHC-linked immune response (Ir) genes (1-4). Nevertheless, antibodies that are stimulated by one species of insulin cross-react extensively with other insulin species (2). Control of responses is conferred by regulatory T cells that recognize differences in amino acid sequences in the A-chain loop of insulin. Although it is clear that certain insulins fail to stimulate antibody responses (1-4) and prime T cells for subsequent proliferative responses in nonresponder mice (5), the cellular mechanisms responsible are not yet clear.

Induction of antigen-specific suppressor T cells is responsible for nonresponsiveness to several other antigens that are regulated by MHC-linked Ir genes including: L-glutamic acid 60-L-alanine 30-L-tyrosine 10 (GAT) (6); hen egg lysozyme (HEL) (7); and lactic dehydrogenase B (LDHa) (8). Therefore, we originally asked whether suppressor T cells regulated responses to insulin using several different experimental approaches. First, we injected nonresponder mice with nonimmunogenic species of insulin and tested their subsequent antibody responses to immunogenic species. Although pork insulin fails to stimulate antibody responses in H-2b mice, prior injection of pork insulin did not inhibit subsequent responses to the normally immunogenic beef insulin (9, 10). Similarly, pork insulin did not inhibit responses to the normally immunogenic pork proinsulin (11) in H-2b mice (J. A. Kapp, unpublished observations). Not only do nonimmunogenic insulins fail to inhibit responses to immunogenic species, but pork insulin actually primes T cells in H-2b mice that provide help to B cells in nonresponder mice.
secondary responses stimulated by beef, but not pork, insulin in vivo (9, 10) and
in vitro (12).

In another attempt to search for suppressor T cells in nonresponder mice
insulin was injected intravenously. We found that T cell tolerance was induced
in responders but not nonresponders by intravenous injection of immunogenic
species of insulin and we could not find any evidence for a role of suppressor T
cells in the induction or maintenance of T cell tolerance (13). Thus, we concluded
that suppressor T cells most likely were not involved in maintaining MHC-linked
nonresponsiveness to insulin.

However, recent experiments by C. W. Pierce et al. (manuscript submitted) demonstrated that nonresponder mice could develop antibody responses to
soluble GAT, if the suppressor T cell pathway was inhibited by removing Lyt-
1+2−, I-J+, Qa-1+ T cells from normal spleen cells before culture initiation. This
observation prompted us to re-evaluate the role of insulin-specific suppressor T
cells in Ir gene control of responses to insulin using a different strategy. The
data presented demonstrate that radioresistant helper T cells specific for pork
insulin were primed by pork insulin in H-2b mice, however pork insulin–primed,
Lyt-1−2+, I-J+ suppressor T cells masked this helper T cell activity. Suppressor
T cells were not primed by beef insulin nor did pork insulin–specific T cells
suppress responses to beef insulin in H-2b mice. Thus, we conclude that nonre-
sponsiveness to pork insulin in controlled by very highly specific suppressor T
cells that prevent the expression, but not the priming of helper T cell activity.

Materials and Methods

Mice. C57BL/10 (B10) mice were bred at the Animal Resources Facility of the Jewish
Hospital of St. Louis, St. Louis, MO. Mice were used at 10–24 wk of age. All mice were
vaccinated with the IHD-I strain of vaccinia virus at 5 wk of age.

Antigens and Immunization. Beef and pork insulins were purchased from Elanco
Products Co., Indianapolis, IN. Fowl γ-globulin (FGG) was purified from chicken serum
obtained from Colorado Serum Co. (Denver, CO). Keyhole limpet hemocyanin (KLH)
was purchased from Calbiochem-Behring Corp., San Diego, CA. Fluorescein (FL) was
coupled to insulin, FGG, or KLH as described (12). Sheep erythrocytes (SRBC, Colorado
Serum Co.) were prepared for use as antigen in culture as described (14). Cell donors
were immunized with 50 μg antigen in a 1:1 mixture with complete Freund’s adjuvant
(CFA; Difco Laboratories, Detroit, MI) containing Mycobacterium tuberculosis H37Ra, in
0.1 ml volume. B cell donors received an initial intraperitoneal (i.p.) injection of antigen
and were boosted with 20 μg i.p. soluble antigen 3 wk after the initial immunization. Cells
were harvested no less than 8 wk after introduction of CFA into the peritoneal cavity. T
cell donors were injected with 50 μg antigen in CFA in the hind footpads 10–20 d before
culture initiation.

Preparation of Lymphocyte Cell Populations. Splenic and lymph node cells were separated
into B cell and T cell fractions as described (9). Briefly, B cell donors were injected with
0.4 ml of a 1:10 dilution of anti-thymocyte serum 2 d before removal of the spleen. B
cells were enriched by incubation with monoclonal rat anti-Thy-1 (T24/40.7 obtained
from the Salk Institute) and prescreened rabbit serum as a source of complement (C)
(Cedarlane Laboratories, Westbury, NY). In some cases, B cells were enriched by adher-
ence to petri dishes coated with affinity-purified goat anti-mouse Ig (0.75 μg/ml) using a
modification of the technique of Mage et al. (15). T cells were enriched from lymph node

Pierce, C. W., C. M. Sorensen, and J. A. Kapp. T cell subsets regulating antibody responses to
L-glutamic acid-L-alanine-L-tyrosine (GAT) in virgin and immune nonresponder mice (submitted).
lymphocytes by adsorption of B cells onto goat anti-Ig-coated petri dishes; T cells were collected as nonadherent cells. In some experiments, single cell suspensions of lymph node T cells were irradiated with 750 R delivered by a Gamma-Cell-40 157Cs source (Atomic Energy of Canada Ltd., Ottawa, Canada).

Subpopulations of T lymphocytes were isolated by both negative and positive selection techniques. In some experiments, Lyt-1 or Lyt-2 cells were eliminated by incubating T cells with 10 μg/ml arsanilate-conjugated monoclonal mouse anti-Lyt-1 or anti-Lyt-2 at 2 × 10^7 cells/ml for 30 min on ice, followed by washing in phosphate-buffered saline (PBS) containing 5 percent newborn calf serum (NBCS, Flow Laboratories, McLean, VA) and incubating with 10 μg/ml rabbit anti-arsanilate serum and C for 45 min at 37°C. All of these antibodies were purchased from Becton Dickinson and Co., Sunnyvale, CA.

Lyt subpopulations were positively selected by incubation of T cells with monoclonal rat anti-Lyt-1 (53-7.515, ATCC Rockville, MD) or anti-Lyt-2 (53-6.72, ATCC) (1:200 final dilution of hybridoma supernatant fluids at 2.5 × 10^7 cells/ml for 30 min on ice). The treated cells were washed with PBS and adherent and nonadherent populations were recovered after incubation on goat anti-rat Ig-coated petri dishes.

Subpopulations were further subdivided on the basis of I-J phenotype by incubation of nonadherent Lyt-1+ or Lyt-2+ T cells with 1:100 final dilution of supernatant fluid from cultures containing monoclonal mouse anti-I-Jα (WF9.40.5 hybridoma cells; gift from Dr. Carl Waltenbaugh, Northwestern Univ., Evanston, IL) (16) or a mixture of WF9.40.5 and supernatant from a hybridoma that recognizes suppressor T cell factors from several H-2b T cell hybridomas (Ky81) a gift from Dr. Vera Hauptfeld, Washington Univ. School of Medicine, St. Louis, MO) (17) or anti-I-Jβ (WF8.C12.8, also a gift from Dr. Carl Waltenbaugh). I-J positive cells were recovered subsequently by differential adherence to goat anti-mouse Ig-coated petri dishes. Alternatively, I-J+ cells were eliminated by incubation of anti-I-J–treated cells with C at 37°C for 45 min.

Cell Culture. Single cell suspensions were incubated in completely supplemented Eagle's minimum essential medium with 10% fetal calf serum (lot 58101, Reheis Chemical Co., Kankakee, IL, or lot 100361 Hyclone Sterile Systems, Inc., Logan, UT) in 16-mm wells of 24-well tissue culture plates (Linbro Chemical Co., Div. Flow Laboratories, Inc., Hamden, CT) for 5 d at 37°C in an atmosphere of 10% CO2, 7% O2, and 83% N2 (18, 19). Each 0.5 ml culture contained 5 × 10^6 B cells and 2-4 × 10^6 T cells. Optimal concentrations of antigen, 0.2 μg/ml insulin, 0.2 μg/ml FL-insulin, 5 ng/ml FL-FGG, or 2 × 10^7 SRBC culture were added at culture initiation (12). In some cultures, ovalbumin (OVA) obtained from Miles Laboratories (Kankakee, IL) was added as an irrelevant antigen.

Adoptive Transfer. The adoptive transfer of secondary insulin-specific antibody responses to irradiated recipients is detectable 7–10 d after challenge and is absolutely dependent upon antigen-primed T cells and B cells (9, 10). Lymph node T cells from mice primed with 50 μg pork insulin in CFA and B cells from spleens of donors primed and boosted with beef insulin were purified as described above. Cells were injected intravenously into syngeneic, sex and age-matched mice that had been irradiated with 650 R. Mice were challenged 24 h after transfer with 50 μg i.p. of beef or pork insulin in CFA.

Hemolytic Plaque Assay. Beef insulin was coupled to SRBC by the method of Schroer et al. (20) and FL was coupled to SRBC by the method of Calderon et al. (21) using fluorescein isothiocyanate (FITC). Insulin or FL-specific indirect plaque-forming cells (PFC) and direct anti-SRBC PFC responses were enumerated after 5 d of culture by the slide modification of the Jerne hemolytic plaque assay (14, 18, 19). IgM plaques were inhibited with goat and anti-mouse μ-chain serum (Gateway Immunonosera, Cahokia, IL) and indirect plaques were facilitated by rabbit anti-mouse IgG serum at a 1/150 dilution. Insulin-specific PFC were determined by enumerating PFC on beef insulin–coupled SRBC and subtracting the number of PFC detected in the presence of 100 μg/ml beef insulin; FL-specific PFC responses were determined by inhibition with 25 μg/ml FL-coupled to E-amino-n-caproic acid (FL-EACA) (12). Duplicate cultures were pooled for PFC determination and data were expressed as PFC/culture. Each experiment has been performed
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several times and since the results are highly reproducible, representative experiments are shown.

ELISA. In some experiments, serum antibodies were determined using a modification of the ELISA described by Engvall and Perlmann (22). Polyvinyl microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 10 μg/ml beef insulin in PBS for 2–4 h at room temperature and excess free-protein binding sites on the plastic wells were saturated with PBS containing 1 mg/ml bovine serum albumin and 0.05% Tween 20 for 1–2 h. Plates were washed with saline and incubated overnight with sera that had been serially diluted in PBS containing 0.05% Tween 20. The plates were washed and incubated for 1½ h at room temperature with alkaline phosphatase coupled to goat anti-mouse immunoglobulin antibody. The plates were washed again and incubated for 30 min at room temperature with 0.6 mg/ml p-nitrophenyl phosphate in buffer containing 1.0 M diethanolamine and 0.2 mM MgCl₂. The O.D. at 405 nm was read on a Microelisa Auto Reader (Dynatech Laboratories). Controls included normal mouse serum, which gave negligible readings, and serum from hyperimmunized mice as a positive control to standardize the assay. This assay is very sensitive and binding could usually be detected at dilutions of ≥1/10,000 in sera from responder mice primed with 50 μg beef insulin in CFA on day 14.

Results

Demonstration of Radiosensitive Pork Insulin–specific Suppressor T Cells. We have recently described conditions under which reliable in vitro secondary antibody responses to insulin can be generated. Development of insulin-specific antibody responses in vitro is absolutely dependent on primed lymph node T cells and primed splenic B cells; using these conditions all of the major experimental observations made in our laboratory concerning genetically controlled immune responses to insulins in vivo are reproducible in vitro (12). Thus, normal regulatory mechanisms appear to be intact in this culture system. Mice of the H-2b haplotype respond in vivo to beef but not to pork insulin; this pattern of responsiveness is conserved in vitro (12). However, previous experiments had shown that H-2b mice primed with pork insulin possessed T helper, (Th) cells capable of stimulating secondary antibody responses of beef but not pork insulin (9, 10, 12). This observation presented a paradox in that these Th cells recognized pork insulin during activation but not during expression of help. To investigate this phenomenon further, pork or beef insulin–primed lymph node T cells were added to cultures containing insulin-primed splenic B cells and various antigens. Although both beef and pork insulin–primed T cells were able to stimulate insulin-specific PFC responses in the presence of beef insulin, neither population appeared to recognize pork insulin (Table I).

The data from this representative experiment also demonstrate that helper activity was radioresistant and that the pattern of response to beef and pork insulin was conserved when beef insulin–primed T cells were irradiated before addition to culture. However, a different result was observed after pork insulin–primed T cells were irradiated. As with beef insulin–primed T cells, helper activity for a response to beef insulin was radioresistant. Interestingly, irradiation of pork insulin–primed T cells unmasked help for a secondary response to pork insulin. Furthermore, addition of unirradiated pork insulin–primed T cells to cultures containing irradiated pork insulin–primed T cells specifically suppressed the response to pork but not beef insulin. Thus, immunization of H-2b mice with pork insulin results in the generation of a radioresistant Th cell population that
TABLE I

**Pork Insulin Primes Radioresistant Helper T Cells and Radiosensitive Suppressor T Cells in C57BL/10 (H-2b) Mice**

| 5 x 10^6 B cells | 2 x 10^6 T cells* | Insulin-specific PFC/Culture† |
|------------------|-------------------|-------------------------------|
|                  | Beef insulin-primed | Pork insulin-primed | No antigen | Beef insulin | Pork insulin |
| Untreated        | 750 R              | Untreated | 750 R |              |               |
| +                | −                  | −         | −     | 20           | 20            | 30           |
| +                | +                  | +         | +     | 35           | 950           | 15           |
| +                | −                  | +         | −     | 40           | 790           | 25           |
| +                | −                  | +         | −     | <10          | 905           | 35           |
| +                | −                  | −         | +     | 25           | 785           | 625          |
| +                | −                  | −         | +     | 25           | 840           | 25           |

* Ig− lymph node lymphocytes from mice primed with 50 μg insulin in CFA in the footpads were used as a source of T cells.
† PFC responses determined on day 5 in cultures stimulated with no antigen or 0.2 μg/ml beef or pork insulin.
‡ Splenic B cells were obtained from mice primed and boosted with beef insulin.

TABLE II

**Ly Phenotype of Pork Insulin-specific Th and Ts Cells**

| 5 x 10^6 B cells | Pork insulin-primed T cells | PFC/Culture* |
|------------------|-----------------------------|--------------|
|                  | Untreated† | Anti-Lyt-2 + C‡ | Anti-Lyt-1 + C§ | No antigen | Beef insulin | Pork insulin | SRBC |
| +                | −         | −         | −         | 10         | 10         | 70          | 2,125 |
| +                | +         | −         | −         | 80         | 1,045      | 90          | 20,525 |
| +                | −         | +         | −         | 100        | 810        | 650         | 24,050 |
| +                | −         | −         | +         | <10        | <10        | <10         | 2,000 |
| +                | −         | −         | +         | 130        | 1,065      | 60          | 20,000 |

* PFC responses on day 5 in cultures stimulated with no antigen, 0.2 μg beef or pork insulin, or SRBC.
† 2.5 x 10^6 untreated pork insulin-primed lymph node T cells.
‡ 1.6 x 10^6 pork insulin-primed T cells treated with anti-Lyt-2 plus C.
§ 0.9 x 10^6 pork insulin-primed T cells treated with anti-Lyt-1 plus C.

provides help in secondary responses to both beef and pork insulins, but responses to pork insulin are normally inhibited by specific, radiosensitive T suppressor (Ts) cells.

**Lyt Phenotype of Pork Insulin-primed Th and Ts Cells.** The Lyt phenotype of pork insulin-primed Th and Ts cells was evaluated by testing the biological activity of residual T cells after incubation with cytotoxic monoclonal anti-Lyt-2 or anti-Lyt-1 antibodies and C in secondary PFC responses to both beef and pork insulin (Table II). As previously shown, untreated pork insulin-primed T cells provide help for responses to beef but not pork insulin. Treatment of pork insulin-primed T cells with anti-Lyt-2 antibodies and C, like exposure of 750 R, permitted the emergence of help for responses to pork insulin. Treatment of pork insulin-primed T cells with monoclonal anti-Lyt-1 plus C abrogated all helper activity. These remaining Lyt-1-2+ cells were, however, able to suppress responses to pork but not beef insulin when added to cultures containing helper T cells treated with anti-Lyt-2 antibody plus C (Lyt-1+2- cells). These observa-
tions suggest that the pork insulin-primed Th cells bear the Lyt-1+2- phenotype of classical primed helper T cells and the pork insulin–specific Ts cells bear the Lyt-1-2+ phenotype.

These results were confirmed using positive selection techniques (Table III). Pork insulin–primed T cells were separated into Lyt-1+ and Lyt-2+ subpopulations by differential adherence to goat anti-mouse Ig-coated petri dishes after incubation with monoclonal rat anti-Lyt-2 immunoglobulin. Helper activity was localized to the Lyt-2- (Lyt-1+) subpopulation and suppression was associated with Lyt-2+ T cells. In addition, the radiosensitivity of the suppressor population was confirmed.

Role of Ts Cells In Vivo. Before further characterization of the pork insulin–primed Ts cells, we wanted to determine whether removal of Lyt-1-2+ T cells from pork insulin–primed T cells would permit responses to pork insulin in adoptive recipients in vivo. One such experiment is shown in Fig. 1. Untreated lymph node T cells from pork insulin–primed mice reconstituted responses by beef insulin–primed B cells when challenged with beef insulin but not pork insulin as previously reported (9). Elimination of Lyt-2+ Ts cells from pork insulin–primed T cells revealed that pork insulin–primed Th cells could reconstitute serum antibody responses to pork insulin in adoptive recipients as detected by ELISA. This experiment and others indicate that pork insulin–specific Ts cells are demonstrable in vivo as well as in vitro, thereby ruling out the possibility that this phenomenon is a tissue culture artifact. Since far fewer cells and mice are required for in vitro assays, the remainder of the experiments were performed in vitro.

Pork Insulin–Specific Ts Cells Must Be Primed. To evaluate the requirement for antigen in the activation of Ts cells, Lyt-2+ T cells from mice primed with pork insulin, beef insulin, or FGG were added to cultures containing pork insulin–primed Lyt-1+ Th and insulin-primed B cells. The only cells capable of suppressing the response to pork insulin were Lyt-2+ T cells from pork insulin–primed mice (Table IV). Beef insulin failed to stimulate cross-reactive Ts cells.

### Table III

| 5 × 10^6 B cells | Pork insulin–primed T cells* | PFC/Culture† |
|------------------|-----------------------------|-------------|
|                  | 3 × 10^6 Un-                 |             |
|                  | treated                     |             |
|                  | 2 × 10^6 Lyt-1+             |             |
|                  | 1 × 10^6 Lyt-2+             |             |
|                  | No antigen                  |             |
|                  | Beef                       |             |
|                  | insulin                     |             |
|                  | Pork                       |             |
|                  | insulin                     |             |
|                  | SRBC                       |             |
| +                | -                          | -           |
| +                | +                          | -           |
| +                | -                          | +           |
| +                | -                          | -           |
| +                | -                          | -           |
| +                | +                          | +           |
| +                | +                          | +           |
| +                | +                          | +           |

* Lymph node T cells from pork insulin–primed mice were either untreated or treated with anti-Lyt-2 antibody and separated into nonadherent (Lyt-1+) or adherent (Lyt-2+) cells by incubation in plates coated with goat anti-rat Ig antibodies.

† See footnotes for Table II.

‡ Lyt-2+ cells were irradiated with 750 R before addition to culture.

NT, not tested.
FIGURE 1. Demonstration of Ts cells in vivo. Lymph node T cells (20 X 10⁶) or Lyt 1⁺ lymph node T cells from C57B1/10 mice primed with 50 µg pork insulin were mixed with B cells (30 X 10⁶) from C57BL/10 mice primed with beef insulin and transferred into irradiated syngeneic mice. Recipient mice were injected with 50 µg beef insulin or 50 µg pork insulin and secondary antibody responses were measured by ELISA. The titers were determined by titration and the inverse of the dilution causing O.D.₄₅₀ of 0.1 was compared to a standard anti-insulin antibody. The results are expressed as the titer relative to the control x 100.

TABLE IV

| Antigenic Requirements for Stimulation of Pork Insulin–specific Ts Cells |
|---------------------------------------------------------------|
|      | 2 x 10⁶  | 1 x 10⁶ Lyt-2⁺ cells primed with | PFC/Culture |
|      | Pork insulin–primed Lyt-1⁺ | |
|      | None | Beef insulin | Pork insulin | SRBC |
| +    | –    | NT           | <10          | 15  | 1,600 |
| +    | +    | <10          | 735          | 595 | 11,575 |
| +    | +    | Pork insulin | <10          | <10 | 12,125 |
| +    | +    | Beef insulin | <10          | 555 | 275   | 10,675 |
| +    | +    | FGG          | <10          | 455 | 500   | 12,225 |

* Ig⁺ lymph node T cells from pork insulin–primed mice were treated with anti–Lyt-2 antibody, incubated on goat anti-rat Ig–coated plates and the nonadherent cells used as a source of Lyt-1⁺ cells.

* Lyt-2⁺ cells from mice primed with pork or beef insulin or FGG were obtained by treating Ig⁺ lymph node T cells described above and collecting the adherent cells as a source of Lyt-2⁺ cells.

and neither beef nor pork insulin–primed Lyt-2⁺ cells inhibited responses to beef insulin. Thus, Ts cells are exquisitely specific and must be primed in vivo to inhibit secondary responses in vitro.

Requirement for I-J⁺ Cells for Suppression. Suppressor pathways identified in a variety of antigenic systems have been shown to involve T cells that bear determinants encoded by the I-J subregion of the H-2 gene complex (16, 23–26). The I-J phenotype of pork insulin–specific suppressor cells was evaluated next. Pork insulin–primed Lyt-2⁺ T cells were separated into I-J⁺ and I-J⁻ subpopulations using differential adherence to goat anti-mouse Ig–coated petri dishes after incubation with monoclonal anti-I-Jb antibody. Lyt-2⁺ I-J⁺ T cells suppressed the response to pork insulin (Table V) while Lyt-2⁺ I-J⁻ cells were associated with only marginal suppressive activity. In other experiments, treatment of Lyt-2⁺ T cells with cytotoxic monoclonal anti-I-Jb, but not anti-I-J⁺
TABLE V

**I-J Phenotype of Pork Insulin-specific Lyt-1-2* Ts Cells**

| 5 x 10^6 B cells | Pork insulin-primed T cells* | PFC/Culture |
|-------------------|-----------------------------|-------------|
|                   | 2 x 10^6 Lyt-1* | 1 x 10^6 Lyt-2* | 1 x 10^6 I-J* | I-J- |
| +                 | +                 | +             | +             |
| +                 | +                 | +             | +             |
| +                 | +                 | +             | +             |
| +                 | +                 | +             | +             |
| +                 | +                 | +             | +             |
| +                 | +                 | +             | +             |

|                | None | Beef insulin | Pork insulin | SRBC |
|----------------|------|--------------|--------------|------|
|                | NT   | <10          | 30           | 5,700|
| +              | <10  | 590          | 480          | 18,825|
| +              | <10  | <10          | <10          | 5,675|
| +              | <10  | 605          | <10          | 15,500|
| +              | <10  | 440          | <10          | 15,875|
| +              | <10  | 450          | 290          | 10,750|

* Lymph node T cells from pork insulin-primed C57BL/10 mice. Lyt-1+ cells were obtained as the adherent cells after treatment with anti-Lyt-1 antibody and incubation on goat anti-rat Ig-coated plates and Lyt-2+ cells were obtained as the nonadherent cells. Purified Lyt-2+ cells were treated with anti-I-J+ antibody and incubated on goat-anti-mouse Ig-coated plates. Adherent cells were used as a source of I-J+ cells and nonadherent cells were used as a source of I-J- cells.

TABLE VI

**Pork Insulin-primed Lyt-2+ Ts Cells Do Not Require Lyt-1*, I-J* Cells**

| 5 x 10^6 B cells | 2 x 10^6 Lyt-1* | 1 x 10^6 Lyt-2* | 1 x 10^6 I-J* | I-J- |
|-------------------|-----------------|-----------------|---------------|-----|
| +                 | +               | +               | +             |
| +                 | +               | +               | +             |
| +                 | +               | +               | +             |
| +                 | +               | +               | +             |
| +                 | +               | +               | +             |

|                | None | Beef insulin | Pork insulin |
|----------------|------|--------------|--------------|
|                | <10  | 255          | 275          |
| +              | <10  | 355          | 10           |
| +              | <10  | 355          | 440          |
| +              | <10  | 350          | <10          |

* Ig+ pork insulin-primed lymph node cells were used as a source of T cells, which were further fractionated into Lyt-1+ (nonadherent) cells and Lyt-2+ (adherent) cells after treatment with anti-Lyt-2 antibody and incubation on goat anti-rat Ig-coated plates. Some of the Lyt-1+ cells were treated with anti-I-J+ antibodies and C to provide Lyt-1+, I-J- cells.

antibodies, plus C abrogated all suppressive activity (data not shown). Thus, pork insulin-specific suppressor cells are radiosensitive, Lyt-1-2*, I-J* T cells. Furthermore, pork insulin-primed Lyt-1-2* T cells suppressed responses to pork insulin even after removal of Lyt-1* I-J* T cells (Table VI). In these experiments, anti-I-J antibodies were shown to be cytotoxic by their ability to remove suppressive activity from Lyt-1-2* T cells (data not shown). These data suggest that there is no requirement for participation of Lyt-1*, I-J* inducer T cells at this step in the insulin-specific suppressor T cell pathway.

The Activity of Pork Insulin-Primed Th and Ts Cells Requires Hapten-Carrier Linkage. T cells have been shown to collaborate with B cells using at least two different pathways (27–29). Experiments were performed next to determine whether pork insulin-primed Th cells act through classical helper mechanisms or through bystander mechanisms. To distinguish these two possibilities, T cells were added to cultures containing hapten-primed B cells and various antigen(s). As expected, unirradiated pork insulin-primed T cells stimulated secondary responses to FL-beef but not FL-pork insulin (Table VII). Irradiated pork insulin-primed T cells helped secondary responses to both FL-beef and FL-pork
insulin in analogy to experiments performed with insulin-primed B cells (Table I). No FL-specific PFC responses were observed in cultures containing the irrelevant antigen, FL-FGG, even when pork or beef insulin were also present in culture. Thus, pork insulin–primed Th cells are classical helper cells in that they require hapten-carrier linkage to stimulate hapten-specific B cells.

Addition of unirradiated pork insulin–primed T cells into cultures containing irradiated T cells as a source of Th cells suppressed responses to FL-pork but not FL-beef insulin. Furthermore, the FL-specific PFC response to FL-beef insulin was not suppressed even when pork insulin was present in the same culture. This observation suggests that pork insulin primes both Th cells and Ts cells and that responses to pork but not beef insulin are dominated by Ts cells. The observation that Th cells that cross-react with beef insulin can be primed by pork insulin, whereas the activities of these primed Th cells could not be inhibited by pork insulin–specific Ts cells even in cultures containing both beef and pork insulin was very puzzling. One interpretation of this observation is that the pork insulin–primed Th cells can react with beef and pork insulin but the avidity for binding beef insulin is much greater than binding of pork insulin. Thus, Th cells would preferentially bind beef insulin when beef and pork insulin are present in equal concentrations (as in Table VI). Since pork insulin–primed Ts cells appear to require an antigen bridge to mediate suppression, they would not be able to deliver the suppressive signal to Th cells binding beef insulin. To test this interpretation, we asked whether increasing the concentration of pork insulin in cultures of pork-primed Th cells stimulated with a fixed amount of beef insulin (0.1 μg/ml) would induce unresponsiveness. The results (Fig. 2) demonstrate that increasing the concentration of pork insulin abrogated the response of beef insulin, whereas increasing concentrations of beef insulin or other immunogenic forms of insulin (sheep or horse) or addition of the irrelevant antigen, OVA, had no suppressive effect. Furthermore, the suppressive effect mediated by pork insulin is related to the molar ratio of pork to beef insulin rather than the absolute concentration of pork insulin, since increasing the beef insulin concentration to 1.0 μg reversed suppression caused by 1.0 μg pork insulin, but further increasing the concentration of pork insulin to 2.0 μg then abrogated the effect of the higher dose of beef insulin (Table VIII).
FIGURE 2. Suppression of immune responses to beef insulin by pork insulin Ts cells requires the presence of excess pork insulin. Cultures containing T cells from pork insulin-primed mice and B cells from beef insulin-primed mice were stimulated with 0.1 μg beef insulin alone (420 insulin-specific PFC/culture) or 0.1 μg beef insulin plus increasing concentrations of beef, sheep, or pork insulin or OVA. Insulin-specific PFC responses were assayed on day 5.

| 3 × 10^6 Pork insulin-primed T cells | 5 × 10^6 Beef insulin-primed B cells | μg/ml Insulin | Ratio | Insulin-specific IgG PFC/Culture |
|--------------------------------------|--------------------------------------|---------------|-------|----------------------------------|
| +                                    | +                                    | 0.1           | -     | -                               | 890  |
| +                                    | +                                    | 0.1           | 0.1   | 1                               | 890  |
| +                                    | +                                    | 0.1           | 0.5   | 5                               | 725  |
| +                                    | +                                    | 0.1           | 1.0   | 10                              | 965  |
| +                                    | +                                    | 0.1           | 1.5   | 15                              | 800  |
| +                                    | +                                    | 0.1           | 2.0   | 20                              | 740  |
| +                                    | +                                    | 0.1           | -     | 0.1                             | 1    | 860  |
| +                                    | +                                    | 0.1           | -     | 0.5                             | 5    | 295  |
| +                                    | +                                    | 0.1           | -     | 1.0                             | 10   | 190  |
| +                                    | +                                    | 0.1           | -     | 1.5                             | 15   | 185  |
| +                                    | +                                    | 0.1           | -     | 2.0                             | 20   | <10  |
| +                                    | +                                    | 1.0           | 1.0   | 1                               | 790  |
| +                                    | +                                    | 1.0           | 2.0   | 2                               | 750  |
| +                                    | +                                    | 1.0           | -     | 1.0                             | 1    | 835  |
| +                                    | +                                    | 1.0           | -     | 2.0                             | 2    | 230  |

* Ig- pork insulin-primed lymph node cells were used as a source of T cells.
** Ig+ spleen cells from mice primed with beef insulin were used as a source of B cells.
† Ratio of pork or sheep insulin to beef insulin added to culture.

Discussion

These data demonstrate that the failure of pork insulin to stimulate antibody responses in nonresponder H-2^b^ mice is the result of the activation of pork insulin-specific suppressor T cells that prevent the expression of primed, pork insulin-specific helper T cells. Once the Ts cells are removed from pork insulin-primed T cells by irradiation, treatment with anti-Lyt-2 antibodies or treatment with anti-I-J antibodies, T cells are revealed that can help responses to beef and pork insulin. These primed T cells are Lyt-1^-2^, I-J^-, which is consistent with
the helper T cell phenotype described in other systems (25, 26, 31). These Lyt-
1\(^{+}\), 2\(^{-}\), I-J\(^{-}\) helper cells are dependent upon hapten-carrier linkage to stimulate
antibody responses, which is typical of classically defined, cognate helper T cells
(27–29). The fact that these Th cells are radioresistant supports the conclusion
that they are primed, not virgin, T cells (30). Priming of these Th cells by pork
insulin clearly demonstrates that T cells from H-2\(^{b}\) mice recognize pork insulin
in the context of self-Ia. Thus, there is no lesion at the level of antigen
presentation by nonresponder, H-2\(^{b}\) macrophages; nor in the ability of B cells to
produce antibodies to pork insulin; nor is there a blank spot in the insulin-specific
Th cell repertoire. Nonresponsiveness is due to dominant, insulin-specific Ts
cells in H-2\(^{b}\) mice that regulate the functional phenotype of the helper T cell
repertoire. This observation raises the question of whether other nonimmuno-
genic forms of insulin, in addition to pork insulin, can prime helper T cells in H-
2\(^{b}\) mice and whether such Th cells can be demonstrated in nonresponder mice
bearing other H-2 haplotypes. Preliminary evidence shows that beef insulin also
primed Th cells in nonresponder, H-2\(^{k}\), mice and that these Th cells were
inhibited by specific Ts cells. However, the generality of this phenomenon is still
under investigation.

The suppressor T cells that regulate responses to insulin in H-2\(^{b}\) mice required
priming by pork insulin in vivo and pork, but not beef, insulin can activate these
cells in vitro. The pork insulin–primed Ts cells are radiosensitive, Lyt-1\(^{-}\)2\(^{+}\), I-J\(^{+}\)
cells that inhibit responses to pork insulin but are unable to suppress responses
to beef insulin. Lyt-1\(^{-}\)2\(^{+}\), I-J\(^{+}\) effector suppressor T cells have been described in
numerous systems, some of which have been shown to require an Lyt-1\(^{+}\), I-J\(^{+}\)
inducer T cell for activation (25, 26, 31). Whether such inducer T cells are an
active component of this suppressor pathway at an earlier stage is currently
under investigation.

Anti-hapten responses were also suppressed by pork insulin–primed Ts when
responses were stimulated with haptenated pork insulin. Anti-hapten responses
were not inhibited when cultures were stimulated with hapten-linked to irrelevant
antigens even if pork insulin was also present, suggesting that Ts cells also require
covalent linkage of hapten to carrier to provide a bridge for delivery of the
suppressive signal to the target cells. Since pork insulin–primed Ts cells can
distinguish pork from beef insulin, two species of insulin that differ from one
another by two amino acids in the A-chain loop, we assume these T cells recognize
the A-chain loop or determinants in association with the A-chain loop (32).
Furthermore, the amino acid sequence of the A-chain loop in pork insulin is
identical to that of mouse insulin (32), which raises the possibility that the primed
Ts cells may be self-reactive and therefore could play a role in maintaining self-
tolerance. Experiments are currently underway to test this possibility.

These studies also provide some insight into the paradoxical observation that
pork insulin primes T cells in H-2\(^{b}\) mice that provide helper activity to B cells
when restimulated by beef, but not pork, insulin in vivo (9, 10) and in vitro (12).
The data presented here suggest that pork insulin primes T helper cells that
cross-react with beef insulin and that these T cells can be inhibited by pork
insulin–specific suppressor T cells if pork insulin is present to provide an antigen
bridge. Responses to beef insulin are not inhibited by pork insulin–specific T
Suppressor cells because these T cells must recognize pork insulin determinants. Furthermore, the cross-reactive Th cells are not inhibited by pork insulin-specific Ts cells in the presence of equal molar concentrations (0.1 μg/ml) of pork and beef insulin. Nevertheless, increasing the concentration of pork insulin two- to fivefold greater than that of beef insulin results in inhibition of the antibody response. If the absolute concentration of beef insulin is raised to that of pork insulin (1.0 μg/ml), suppression is abrogated but can be reinstated by increasing the ratio of pork to beef insulin twofold. One interpretation of the observation that suppression depends upon the molar ratio between pork and beef insulin rather than the absolute concentration of pork insulin is that the pork insulin–primed T helper cells have a higher avidity for the cross-reacting antigen, beef insulin, than the stimulating antigen, pork insulin. Such phenomena have been observed before and have been called heteroclitic responses (33, 34). Thus, Th cells must bind sufficient pork insulin by recognition of cross-reacting determinants so that pork insulin–specific Ts cells can interact with them to inhibit the response. Other, more complicated explanations for this observation could be envisioned, but this interpretation is compatible with all of the data.

Perhaps the most important message to be taken from the observations reported here concerns the conditions that are necessary to demonstrate active suppression. In most systems in which suppressor cells are known to be involved in the regulation of genetically determined nonresponsiveness, these cells were first demonstrated by cross-reactive suppression of responses to normally immunogenic antigens. Sercarz and associates (7) have shown that preimmunization of nonresponders with HEL induces suppression of anti-TNP antibody responses to otherwise immunogenic, TNP-guanidinylated-hen lysozyme. In the case of GAT, preimmunization of nonresponders with soluble GAT induces T cells that subsequently suppress responses to the normally immunogenic antigen, GAT complexed to methylated bovine serum albumin (GAT-MBSA) (6). However, elimination of GAT-primed Lyt-1−,2+ suppressor T cells still did not permit development of responses to soluble GAT. More recent evidence shows that antibody responses to soluble GAT can develop in unprimed (normal) or GAT-primed nonresponder spleen cells if a radiosensitive, Lyt-1+2−, I-J+ Qa-1+ suppressor cell is also eliminated (Pierce, Sorensen, and Kapp).2 The Ts cells described here do not suppress responses to normally immunogenic, heterologous insulins. An immune response to pork insulin is observed only after amputation of the pork insulin–specific suppressor limb. A similar situation was described by Baxevanis and associate (8) in which T cells from primed nonresponder strains developed proliferative responses to LDHb in vitro only after addition of antibodies that specifically blocked the suppressor pathway or after removal of Lyt-2–bearing Ts cells. Collectively, these studies suggest that H-2–linked Ir gene control of immune responses is the net result of the stimulation of a variety of T cell subsets and that “holes” in the functional repertoire of Th cells can be mediated by the dominant activity of Ts cells. To our knowledge, this general approach has not been used in a number of otherwise well-characterized systems in which genetically determined nonresponsiveness is studied. Thus, suppressor T cells may be more widely involved in the expression of Ir gene control than currently expected.
Summary

Murine antibody responses to insulins are controlled by MHC-linked Ir genes. Although mice of the H-2b haplotype do not make antibody in response to pork insulin, we demonstrate in this communication that immunization with pork insulin stimulates radioresistant, Lyt-1+2- helper T cells that are capable of stimulating secondary antibody responses to pork insulin in vitro, but that this activity is masked by radiosensitive, Lyt-1-2+, I-J+ suppressor T cells. The suppressor T cells, present after immunization with pork insulin but not beef insulin, suppress the secondary response to pork but not beef insulin. The amino acid sequences of pork and beef insulins differ only at the A-chain loop; thus, pork insulin–specific suppressor T cells appear to recognize the A-chain loop determinant of pork insulin. The amino acid sequences of mouse and pork insulin are identical in the A-chain loop, which suggests that these suppressor T cells may be self-reactive. If this interpretation is correct, these suppressor T cells could be involved in the maintenance of self-tolerance to insulin. Nevertheless, these data clearly demonstrate that genetically determined nonresponsiveness in H-2b mice is conferred by activation of dominant, insulin-specific suppressor T cells (Ts), rather than by a defect in the stimulation of insulin-specific helper T cells (Th).

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