Brief Definitive Report

INDUCIBLE EXPRESSION OF INSULIN RECEPTORS ON T LYMPHOCYTE CLONES*

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Insulin receptors are present on lymphoid cell lines (1) and activated murine and human lymphocytes (2, 3) with similar physicochemical properties to those on liver cells and adipocytes. Resting small lymphocytes apparently lack specific binding sites for insulin (2, 3), whereas they are present on mitogen-stimulated human (3) and rat (2) lymphocyte populations. Also, activated murine or human lymphocytes generated in mixed lymphocyte cultures express insulin receptors (4, 5).

Our attention was drawn to the investigation of insulin receptors on T lymphocytes by an intriguing report citing the insulin receptor as a universal marker of activated lymphocytes (2). Because the above studies on lymphocyte insulin receptors have been done on heterogeneous lymphocyte populations, it was of interest to determine whether the insulin receptor is indeed expressed on all the various activated T and B cell subsets, or rather on some but not others. If insulin receptors are expressed by a fraction of activated lymphocytes, can they be used as cell surface markers to define functional lymphocyte subpopulations or used as determinants of differentiation states of a given lymphocyte lineage? To investigate these questions, one can fractionate splenic lymphocytes into subsets based on cell surface markers (e.g., Thy-1, Lyt-1, Lyt-2, surface immunoglobulin) and examine these for insulin receptor expression. Another possibility would be to examine cloned lymphocyte cell lines.

We decided to pursue the latter direction using murine T cell clones, which are H-2 restricted, influenza virus specific and antigen dependent for proliferation (6, 7). We recently reported (6) the isolation, characterization, and continuous propagation of these cloned T lymphocyte lines. The availability of these T cell clones affords the opportunity of examining insulin receptor expression on homogeneous, nontransformed cell lines with cytotoxic or helper function. Also, using a homogeneous T cell clone, we can determine whether the insulin receptor can be a marker of the activated vs. the resting state of a given T cell clone subsequent to antigenic stimulation.

This report describes the expression of insulin receptors on noncytotoxic T lymphocyte clones subsequent to specific antigenic stimulation and the apparent lack of detectable insulin specific receptors on activated cytotoxic T cell clones.

Materials and Methods

T Cell Clones. The isolation, characterization, and propagation of cytotoxic T cell clones 14-1, 14-7, and 14-13 have been previously described (6). Cytotoxic T cell clone 23-9, of

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(BALB/c × C57BL/6) F₁ (H-2<sup>d</sup>/k) origin and H-2<sup>d</sup> restricted in its recognition of A/Memphis/1/71 influenza virus, was isolated and maintained in the same manner as the above cytotoxic T lymphocytes (CTL) clones. Non-CTL clones (22-2, 14-16, 28-3, M3-2, M3-3) were isolated and maintained by the methods described for the CTL clones. Clone 22-2 is of CB6F₁ (H-2<sup>b</sup>/b) origin and H-2 restricted, A/MEM/71 specific. Clone 14-16 is of CB6F₁ origin and H-2<sup>d</sup> restricted in its recognition of A/JAP/57. Clone 28-3 is of BALB/cBy (H-2<sup>a</sup>) origin and is H-2 restricted and A/MEM/71 specific. Clones M3-2 and M3-3 are BALB/cBy (H-2<sup>a</sup>) in origin and are specific for H-2<sup>I</sup> region molecules. Viable cloned T cells were separated from irradiated stimulator cells by centrifugation on Isopaque-Ficoll (8) before use in the insulin radioreceptor assay and indirect rosette assay. (BALB/c × C57BL/6)F₁, BALB/cByJ, C57BL/6J, CBA/J, and CBA/CaJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Influenza virus stocks were grown and stored as previously described (9).

**125I-Insulin Binding Assay.** Porcine insulin and porcine proinsulin were kindly supplied by Dr. R. Chance of Eli Lilly and Company, Indianapolis, IN. Desoctapeptide bovine insulin was a gift from Dr. H. Carpenter. Glucagon was provided by Dr. R. Gingerich (Washington University, St. Louis, MO) and human growth hormone was generously provided by the National Pituitary Agency (Baltimore, MD). 125I-insulin was prepared at specific activities of 100–200 µCi using enzymo-beads from Bio-Rad Laboratories (Richmond, CA) or a soluble lactoperoxidase method (10).

Cells (1–2 × 10<sup>7</sup>) were sedimented, medium aspirated, and pellets were resuspended in buffer containing 25 mM Tris, 120 mM NaCl, 1.2 mM MgSO<sub>4</sub>, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, and 1% bovine serum albumin, pH 7.8. Labeled hormone (10<sup>−11</sup> to 10<sup>−10</sup> M) and cells with and without increasing concentrations of unlabeled hormone were incubated for 90–120 min at room temperature in 1.5 ml polypropylene tubes in a final volume of 0.5 ml. At the end of the incubation, tubes were centrifuged in a Beckman Microfuge (Beckman Instruments, Inc., Fullerton, CA) for 90 s. Supematants were aspirated, and pellets were washed with 0.4 ml of cold buffer. Cells were spun for an additional 30 s, supernatants aspirated, and pellets excised. Cell-associated radioactivity was counted in an autogamma counter. Data were analyzed by methods previously described (11).

**[3H]Thymidine Incorporation.** Cellular proliferation was assessed using a 4-h [3H]thymidine incorporation assay. Quadruplicate 100-µl culture samples were delivered to microtiter plate wells, and 100 µl of [3H]TDR in phosphate-buffered saline (10 µCi/ml) were added per well. Contents of wells were harvested onto glass fiber filters using an automated cell harvester. Results are expressed as the mean counts per minute incorporated of the quadruplicates.

**Lyt Phenotyping.** Viable cloned T cells were incubated with monoclonal anti-Lyt-1 (53-7.313) or monoclonal anti-Lyt-2 (53-6.72) antibodies and used in an indirect rosette assay, which has been described (6).

**Mixed Lymphocyte Culture.** Equal numbers of responder cells from CBA/CaJ mice (5 × 10<sup>7</sup> splenic lymphocytes) and irradiated (2,000 rad) stimulator cells from CBA/J mice were co-cultured in Eagle's minimal essential medium containing 10% fetal bovine serum and 2 × 10<sup>−6</sup> M 2-mercaptoethanol and incubated at 37°C in a 7% CO<sub>2</sub>/air atmosphere.

**Results**

To assess the role of the insulin receptor as a marker for activated T lymphocytes, we examined a panel of cytolytic and noncytolytic murine T cell clones for the expression of insulin receptors 2–4 d after antigenic stimulation—the peak of the proliferative response. The noncytolytic T cell populations were either H-2I region-restricted, influenza virus-specific clones (22-2, 14-16, 28-3) or alloreactive clones directed to H-2I region products (M3-2, M3-3). These T cell clones exhibited no specific lytic activity on target cells in conventional cytotoxicity assays (unpublished results) and lacked the Lyt-2 surface marker (Table I). They can, however, proliferate in response to specific antigenic stimulation in the absence of exogenous T cell growth factor (unpublished observations). As Table I demonstrates, radiolabeled insulin bound to all of these cloned T cell populations in a specific fashion, indicating the
Table I

| CTL line     | Day of assay* | Lyt phenotype‡ | Percent displaceable counts§ | Percent specific binding¶ |
|--------------|---------------|----------------|-------------------------------|--------------------------|
| Noncytotoxic T clones |               |                |                               |                          |
| 22-2         | 3             | 1⁺ (76) 2⁻     | 70.8                          | 8.4                      |
| 14-16        | 3             | 1⁻ 2⁺          | 75                            | 3.8                      |
| 28-3         | 3             | 1⁺ (48) 2⁻     | 81                            | 8.0                      |
| M3-2         | 3             | 1⁺ (66) 2⁻     | 75                            | 3.13                     |
| M3-3         | 3             | 1⁺ (44) 2⁻     | 71                            | 4.46                     |
| Mls bulk     | 3             |                | 86                            | 8.4                      |
| 22-2         | 11            |                | 38                            | 0.93                     |
| Cytotoxic T clones |         |                |                               |                          |
| 14-1         | 2             | 1⁺ (37) 2⁻     | 5                             | 0.19                     |
| 14-1         | 4             |                | 1.5                           | 0.07                     |
| 14-1         | 10            |                | 0.03                          | 0.02                     |
| 14-7         | 2             | 1⁺ (16) 2⁻     | 0.00                          | −0.21                    |
| 14-13        | 4             | 1⁺ (50) 2⁻     | 18                            | 0.12                     |
| 23-9         | 4             | 1⁺ (13) 2⁻     | 0.00                          | −0.04                    |

* Indicates days post-antigenic stimulation.
‡ Numbers in parentheses indicate percent rosette positive cells.
§ Represents percent of total counts bound displaced by \(10^{-6}\) M insulin. Positive (i.e., receptor-bearing) cells were required to demonstrate >70% displaceable counts.
¶ Represents total counts bound per 10⁶ viable cells minus nonspecific binding (i.e., radioactivity bound in presence of \(10^{-6}\) M insulin).

expression of specific insulin receptors on these activated T cells. In contrast, a panel of H-2-restricted, influenza virus-specific CTL clones, stimulated and maintained under identical conditions, showed only background levels of specific insulin binding in the standard ligand displacement assay (Table I). The level of specific insulin bound by several noncytolytic T cell clones was comparable to that demonstrated by a heterogeneous population of Mls⁴ locus reactive T cells from a mixed lymphocyte culture (CBA/CaJ (Mls⁵) responders and irradiated CBA/J (Mls⁶) stimulators) (Table I).

Table II summarizes studies on the insulin receptor specificity of a representative noncytolytic T cell clone. The potency of various insulins, insulin derivatives, and noninsulin peptides to inhibit porcine \(^{125}\)I-insulin binding to the cloned T lymphocytes is virtually identical to that seen for IM-9 human lymphoid cells. In previous studies, it was shown that IM-9 insulin receptors have specificity for binding that is identical to that observed in liver membranes or isolated fat cells (1).

During the course of this study, representative cytolytic and noncytolytic T cell clones were examined for insulin receptor expression at various times after antigenic stimulation. The cytolytic clone 14-1 showed no specific insulin binding at days 2, 4, and 10 after antigenic stimulation (Table I). In contrast, the noncytolytic clone 22-2, which expressed the insulin receptor on day 3 after antigenic stimulation, was negative for receptor expression by day 11 (Table I). In light of this observation, it was of interest to examine the kinetics of receptor expression on the noncytolytic T cells after antigenic stimulation. Using clone 22-2, we examined the emergence of insulin receptors, the incorporation of \(^{3}H\)thymidine as a measure of rate of proliferation, and viable cell numbers at days 2, 3, 4, and 9 post-antigenic stimulation. As seen in Fig. 1, resting T cells (day 0) lack detectable insulin receptors. After antigenic stimulation, both the expression of insulin receptors (panel a) and \(^{3}H\)thymidine incorporation in the cultures (panel b) peak on day 3 and decline by day 4. The cell numbers (panel c) are still increasing as of day 4 but slowly decline by day 9 because...
TABLE II
Specificity of T lymphocyte Insulin-binding Receptor

| Preparation            | IM-9 cell Potency relative to porcine insulin* | T cell clone 22-2 Potency |
|------------------------|-----------------------------------------------|---------------------------|
| Porcine insulin        | 100                                           | 100                       |
| Porcine proinsulin     | 5.6                                           | 10                        |
| Catfish insulin‡       | 24                                            | 15                        |
| Desoctapeptide insulin | 1.3                                           | 1.5                       |
| Glucagon               | 0                                             | 0                         |
| Human growth hormone   | 0                                             | 0.14                      |

* Potency estimates expressed as the molar concentration of porcine insulin required for 50% inhibition of specific binding/molar concentration of peptide for 50% inhibition of binding × 100.

‡ Catfish insulin supplied by Dr. M. A. Permutt. (Washington Univ., St. Louis, MO.)

Fig. 1. Induction of insulin receptor expression (panel a), rate of proliferation (panel b), and cell growth (panel c) for noncytotoxic T cell clone 22-2. Clone 22-2 cells (day 10 post-antigenic stimulation) were cultured in the presence of A/Memphis/1/71 influenza-infected, irradiated (2000 rad) syngeneic (CB6F1) splenic stimulators and medium with 10% crude rat concanavalin A supernatant (day 0). At various days (2, 3, 4, 9), cells were harvested for the insulin radioreceptor assay (panel a), [3H]thymidine incorporation assay (panel b), or trypan blue exclusion viable cell counts (panel c). Scatchard analysis of binding data on day 3 revealed a $K_A$ of $5.65 \times 10^5 \text{ M}^{-1}$, similar to the insulin receptors of various biologically responsive tissue (14).

Discussion

In this report we have examined a panel of murine T lymphocyte clones with different functional activities (cytolytic vs. noncytolytic) for the expression of insulin...
receptors. We have previously reported (6) the isolation and characterization of long-term H-2-restricted virus-specific CTL clones. By a similar protocol, we have isolated noncytotoxic T cell clones that are antigen specific, and although grown in the presence of exogenous T cell growth factor, do not require it (12, unpublished observations). Our goals in this report were first, to examine our T cell clones for insulin receptor expression, and second, to correlate insulin receptor expression with events after specific antigenic stimulation.

The noncytotoxic T cell clones we have examined, which express insulin receptors, do so in an inducible fashion. That is, insulin receptors are detectable shortly after antigenic stimulation but are absent several days later when the cells are still viable but no longer actively proliferating as measured by \[^{3}H\]thymidine incorporation. These resting cells then readily express insulin receptors again when stimulated by specific antigen.

In contrast, the cytotoxic T cell clones that we have examined lack detectable insulin receptors, both shortly after antigenic stimulation (at the peak of the proliferative response) and at various times thereafter. This result was somewhat surprising in light of the finding that insulin could augment the in vitro activity of in vivo allograft stimulated lymphocytes (13) and data indicating a close relationship between cytotoxic T effectors and insulin receptor-bearing lymphocytes from the same mixed lymphocyte culture (5). It should be emphasized that the dichotomy between noncytolytic and cytolytic T cell clones in insulin receptor expression cannot be attributed to culture conditions, as both the cytotoxic and noncytotoxic T cell clones used in these experiments were grown in the presence of antigen and exogenous T cell growth factor (concanavalin A supernatant).

Our results suggest that the insulin receptor is not as universal a marker of T cell activation as previously thought. It seems likely that there may be discrete T cell subpopulations in which insulin will have biologic effects, whereas the equivalent effects on other T cell subpopulations may well be regulated by other insulin-like peptides.

We are, therefore, now engaged in investigations on the effects of insulin on the growth and function of the cytotoxic vs. non-cytotoxic T cell clones. Also, we are currently screening human cytotoxic and noncytotoxic T cell clones for insulin receptor expression. The availability of these homogeneous nontransformed cells with functional activity and inducible insulin receptor expression opens up new opportunities to study the effects of insulin on the immune system and also its effects on lymphocyte cellular function and growth.

Summary

This study demonstrates that the insulin receptors are expressed on the surface of some T cell clones after specific antigenic stimulation. The insulin receptors on these lymphocytes are physicochemically similar to insulin receptors present on cells which express the receptors constitutively (adipocytes, hepatocytes, etc.). The kinetics of expression of insulin receptors on cloned, noncytotoxic T cells after specific antigenic stimulation closely parallels that of \[^{3}H\]thymidine incorporation in such cultures.

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