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### **Research Article**

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# Rapid Mixed Lymphocyte Culture Testing by Analysis of the Insulin Receptor on Alloactivated T Lymphocyte

## IMPLICATIONS FOR HUMAN TISSUE TYPING

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**ABSTRACT** Responses in the mixed lymphocyte culture (MLC) are traditionally evaluated by measurement of DNA synthesis or blast transformation. However, these events occur too late in the MLC to permit prospective matching for cadaveric renal transplantation. Presentation of allogeneic cells to the T lymphocyte within the MLC results in the emergence of an insulin receptor pharmacokinetically similar to that on other tissues such as fat, liver, and muscle. Intrafamilial MLC were studied by simultaneous assessment of DNA synthesis and insulin receptor binding. In 68 studies from seven families that provide examples of two haplotype identical matches, haplo-identical matches and total haplo mismatches, the presence of an insulin receptor correlated in every case with a positive MLC as estimated by [<sup>3</sup>H]thymidine incorporation. A quantitative relationship existed between the strength of the MLC and the amount of receptor binding. Based on analysis of cells from several families in which cross-over events were known to have occurred, the appearance of an insulin receptor always corresponded with a mismatch at the portion of histocompatibility leukocyte antigen (HLA) chromosome bearing the D region. Finally, it was demonstrated in each of 30 cultures that insulin receptor emergence occurred significantly before detectable DNA synthesis, as early as 24 h after the initiation of the MLC, well within the time-constraint limitations for renal preservation. Appearance of the insulin receptor on activated lymphocytes may be a more rapid measure of mixed lymphocyte

responses, and should permit prospective matching for cadaveric renal transplantation.

## INTRODUCTION

Gene products encoded in the histocompatibility leukocyte antigen (HLA) region of chromosome six have various functions in transplantation immunity. Transplantation outcome may relate to matching entire haplotypes or specific regions rather than solely HLA-A and B antigens (1). Weak, albeit positive, mixed lymphocyte stimulation is predictive of successful engraftment, while strong stimulation is correlated with early graft loss in one-haplotype-mismatched, intrafamilial renal transplants (1-6). Unfortunately, the method by which lymphocyte reactivity in the mixed lymphocyte culture (MLC)<sup>1</sup> is detected requires 4-6 d of tissue culture. This culture time precludes its use for prospective matching of a cadaveric allograft because of the present limitations ( $\leq 72$  h) on organ preservation. We report here the ability to use the rapid appearance of an insulin receptor upon allostimulated T lymphocytes as a measure of MLC alloreactivity for clinical and research endeavors.

## METHODS

**Cell preparation.** Blood was collected into sterile, heparinized tubes. Peripheral blood mononuclear leukocytes collected by Ficoll-Hypaque density gradients from each subject (7) were divided into two aliquots. One aliquot received either 2,000 rad for 20 min or 50  $\mu$ g/ml of mitomycin C for 30 min followed by wash to inhibit DNA synthesis. The other cell population was not treated and served as the responder population in unidirectional MLC. In each experi-

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<sup>1</sup> Abbreviation used in this paper: MLC, mixed lymphocyte culture.

ment, nonstimulatory as well as stimulatory cultures were provided encoded by a nonparticipant in the study to provide internal positive and negative controls. MLC were established by mixing  $10^5$  lymphocytes from a responder subject with  $10^5$  lymphocytes from a stimulator subject in RPMI 1640 medium (Microbiological Associates, Walkersville, Md.) supplemented with 10 mM Hepes and 10% normal, heat-inactivated human serum in round-bottom microtiter trays in an atmosphere of 5% CO<sub>2</sub> and air. Simultaneously, at least  $10^7$  lymphocytes of the same responder and stimulatory pairs were placed in macrocultures in 20 ml of culture medium. All macrocultures were harvested by passage over nylon wool prepared by the method of Handwerger and Schwartz (8) to eliminate the majority of macrophages from the insulin-binding assay. This method permitted recovery of at least 60% of the lymphocytes from the original culture and always permitted the recovery of at bottom the  $4 \times 10^6$  cells minimally required for the binding assay to be performed in duplicate. Elimination of lymphocyte contamination by macrophages is crucial, as macrophages but not lymphocytes freshly prepared from human blood express insulin receptors (9). The technique for exclusion of measurable insulin receptor-bearing residual macrophages was adequate, as no radiolabeled insulin binding was detected in syngeneic cultures.

In the first set of experiments, cultures were harvested at 6 d, as preliminary experiments indicated this time to be that at which peak allostimulated DNA synthesis would be observed. Aliquots of cells from cultures were analyzed both for incorporation of [<sup>3</sup>H]thymidine and for insulin receptor binding.

**Tissue typing.** HLA A, B, and C typing was carried out by the microdroplet complement-dependent cytotoxicity assay (10). Properdin Factor B allotypes were recognized as described by Raum and co-workers (11). DR typing was performed with typing sera of known specificity as defined in the 7th International or 3rd American Workshop using methods previously described (12).

**Interpretation of a positive MLC.** Quantitation of DNA synthesis was used as a measure of the strength of reaction in the MLC. 1  $\mu$ Ci of [<sup>3</sup>H]thymidine was added to each MLC well 18 h before harvest by filtration through glass fiber filters by the Mash II Cell Harvester (Microbiological Associates). The filters were conserved, placed in a standard PPO/POPOP fluor, and counted in a liquid scintillation spectrophotometer. A positive MLC was judged as that culture giving a stimulation index  $\geq 2$ , where the stimulation index is the ratio of the counts per minute from the experimental MLC to an autologous control after subtraction of background counts from each.

*Time course of human lymphocyte culture responses and*

*insulin receptor appearance.* To ascertain the kinetic relationship between the stimulation of DNA synthesis in the MLC and the emergence of a lymphocyte insulin receptor, 400 ml of blood was withdrawn from two subjects who were known to be mismatched for the D locus. A series of macro-MLC was established as described above. Aliquots of these cultures were percolated through nylon wool on each of 10 d after initiation of the cultures. The cultures were assessed for DNA synthesis and for insulin receptor binding.

To confirm the rapid appearance of the lymphocyte insulin receptor detected by the initial time-course studies, 30 additional MLC were established and harvested for the insulin receptor assay after passage of the cells through nylon wool columns 24 h after culture initiation. In these cultures,  $1.5\text{--}10 \times 10^6$  cells from each member of the combination were placed in  $12 \times 75$ -mm Falcon tissue culture tubes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) in 2 ml of RPMI 1640 buffered with 10 mM Hepes and supplemented with 5% decompartmented normal human serum, 2 mM glutamine, and 50  $\mu$ M 2-mercaptoethanol. The enriched medium was chosen to maximize potential alloreactivity in the MLC. There were 6 cultures between subjects sharing no HLA antigens (total mismatches), 16 between related subjects sharing one HLA haplotype, and 8 between related subjects who are HLA identical.

**Insulin receptor assay.** The basic assay technique was a modification of that described by Gammeltoft and Gliemann (13) and Gliemann et al. (14), and has been extensively described and validated in previous publications (15, 16). Our modifications permitted sedimentation of lymphocytes through an oil density gradient at such a specific gravity that 96% of the lymphocytes were recovered in the pellet and contamination of the pellet by free insulin was  $<0.04\%$ . This technique determined both specific and nonspecific binding by virtue of competitive protein binding between <sup>125</sup>I-insulin and unlabeled porcine insulin during a 45-min incubation period at room temperature. The cell mixture was placed upon a bed of phthalate oils in a plastic Beckman microfuge tube (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and spun rapidly for 2 min. The bottom portion of the plastic tube containing the cell pellet was cut with a razor blade and reserved for counting in a gamma spectrophotometer.

## RESULTS

68 MLC combinations were established between individuals from seven families and harvested after 6 d of tissue culture. In those MLC in which there were two HLA haplotype mismatches present (Table I), the

TABLE I  
Radioreceptor Insulin Binding and the Magnitude of  
MLC Analyzed at 1 and 6 D

| No. of haplotype mismatches | 1 d                      |                 |                       | 6 d                      |                 |                       |
|-----------------------------|--------------------------|-----------------|-----------------------|--------------------------|-----------------|-----------------------|
|                             | No. of cultures          | Insulin binding | MLC stimulation index | No. of cultures          | Insulin binding | MLC stimulation index |
|                             | pg/10 <sup>6</sup> cells |                 |                       | pg/10 <sup>6</sup> cells |                 |                       |
| 2                           | 6                        | 5.3±0.7         | 1.2±0.2               | 37                       | 6.8±1.3         | 45±7                  |
| 1                           | 16                       | 3.2±0.4         | 1.3±0.1               | 21                       | 3.4±1.0         | 22±5                  |
| 0                           | 8                        | 0.1±0.1         | 0.9±0.1               | 10                       | 0.1±0.2         | 1.1±0.2               |

Values are mean  $\pm$  SEM.

insulin receptor assay ( $6.8 \pm 1.3$  pg/ $10^6$  cells, range 2.8–9.6) and thymidine assays (SI  $45 \pm 7$ , range 18–115) were positive. In 20 of 21 MLC with but one shared HLA haplotype, both assays were again positive, but with less radioreceptor binding and a lower SI generally ( $3.4 \pm 1.0$  range 1.6–9.4 pg/ $10^6$  insulin bound; SI  $22 \pm 5$ , range 11–48). The 10 MLC between HLA-identical sibs were negative by both assays ( $0.1 \pm 0.2$  pg/ $10^6$ ; SI  $1.1 \pm 0.2$ ). In no case did a single MLC without an HLA mismatch give binding  $\geq 0.5$  pg/ml, a value that represents 1 SD below the mean binding for positive MLC when the outcome is known. Furthermore, the insulin-binding assay performed on cells from the autologous MLC used to provide the denominator in the calculation of the stimulation index was negative in each of the 98 cases studied here. In 1 of 60 studies, a false negative was appreciated by both radioreceptor binding and thymidine uptake. Repeat culture between the same individuals was positive by both methods of assessment.

We can conclude from these family studies that the insulin receptor assay is generally positive when there is an HLA haplotype mismatch and that the receptor assay is uniformly concordant with MLC responses assessed by [ $^3$ H]thymidine. Thus, the generation of the insulin receptor on alloactivated lymphocytes is linked to the major histocompatibility complex and can be predictive of MLC responses.

*Insulin receptor emergence is correlated with mismatches at the HLA-D locus.* To study the immunogenetic relationships between alloantigenic stimulation assessed by thymidine incorporation and the insulin radioreceptor assay, MLC between sibs from three separate families including members with HLA region recombinations were performed. The genotype assignments for each member of the three families, including members with intra-HLA recombinant events, is detailed in Table II. The cells harvested from the MLC between sib T and P in family 1 (Table III), subjects who shared the paternal haplotype and the B and D alleles on the maternal haplotype, did not have a significant SI, nor was specific insulin binding detected. Thus the HLA-A locus does not govern receptor appearance in the MLC. In contrast, MLC-activated cells using D and P (sibs in family 1 sharing only the HLA-A allele on the maternal haplotype and mismatched for other HLA regions) were positive by both assays. This study demonstrated that generation of insulin receptors on responder cells is stimulated by histoincompatible alleles to the centromeric side of HLA-A. MLC in family 2, mounted between sibs B and D in a family containing a member with a genetic recombination between HLA-D and properdin Factor B (also located on the sixth chromosome [11]), demonstrated that when HLA-A, -B and -D were mismatched but matched for Bf, both radioreceptor and thymidine

TABLE II  
HLA Genotypes in Recombinant Families

| Family | Recombination   |  |
|--------|---|--|
| 1      | A-B   |  |
| Father | Aw32 Bw44 DR4/Aw24 B13 DRX                                    |  |
| Mother | Aw24 Bw35 DR5/A28 Bw51 DR7                                    |  |
| T      | Aw32 Bw44 DR4/Aw24 Bw35 DR5                                   |  |
| D      | Aw32 Bw44 DR4/A28 Bw51 DR7                                    |  |
| P      | Aw32 Bw44 DR4/A28 <u>Bw35 DR5*</u>                            |  |
| 2      | D-Bf  |  |
| Father | A10 B40 DR7 Bf <sub>s</sub> /A2 Bw35 DR3 Bf <sub>s</sub>      |  |
| Mother | A1 B17 DR2 Bf <sub>s</sub> /A2 B15 DR4 Bf <sub>r</sub>        |  |
| B      | A2 Bw35 DR3 Bf <sub>s</sub> /A1B17 DR2 Bf <sub>s</sub>        |  |
| D      | A10 B40 DR7 Bf <sub>s</sub> /A1 B17 DR2 Bf <sub>s</sub>       |  |
| P      | A10 B40 DR7 Bf <sub>s</sub> /A2 B15 DR4 <u>Bf<sub>s</sub></u> |  |
| 3      | B-DR  |  |
| Father | A2 B8 DR3/A11 B12 DR4   |  |
| Mother | A2 B12 DR1/Aw31 B5 DRw6                                       |  |
| B      | A2 B8 DR3/A2 B12 DR1  |  |
| G      | A2 B8 DR3/Aw31 B5 DRw6  |  |
| K      | A2 B12 DR1/A11 B12 DR4  |  |
| S      | A2 B12 <u>DRw6</u> /A11 B12 DR4                               |  |

\* The underlined alleles represent the observed recombinations.

assays were positive. Thus, insulin receptor generation must be stimulated by histoincompatible genes residing between HLA-B and Bf. The MLC between K and S in family 3, containing a member who has inherited a recombination between HLA-B and -D pro-

TABLE III  
Comparison between [ $^3$ H]Thymidine Uptake and Insulin Receptor Assay in MLC from Informative Recombinant Pairs

| MLC pair              | Mismatched alleles |   |   |    | [ $^3$ H]Thymidine |       | Insulin binding  |
|-----------------------|--------------------|---|---|----|--------------------|-------|------------------|
|                       | A                  | B | D | Bf | Uptake             | SI    |                  |
|                       |                    |   |   |    | cpm                |       | pg/ $10^6$ cells |
| Family 1              |                    |   |   |    |                    |       |                  |
| T plus P <sub>m</sub> | +                  | – | – | ×  | 721                | 1.2   | 0.2              |
| D plus P <sub>m</sub> | –                  | + | + | ×  | 26,444             | 37.0  | 5.4              |
| Family 2              |                    |   |   |    |                    |       |                  |
| B plus P <sub>m</sub> | +                  | + | + | –  | 38,266             | 55.0  | 8.3              |
| D plus P <sup>m</sup> | +                  | + | + | –  | 37,897             | 51.0  | 8.8              |
| Family 3              |                    |   |   |    |                    |       |                  |
| K plus S <sub>m</sub> | –                  | – | + | ×  | 61,474             | 115.0 | 11.1             |

SI, stimulation index, calculated as the ratio of the counts per minute from the MLC pair to that of an autologous culture. +, allelic difference; –, no allelic difference; ×, untyped allele.

viding a mismatch at the D region, led to the generation of the insulin receptor on allostimulated lymphocytes. Taken together, these studies identify the HLA-D region as responsible for stimulating insulin receptor generation.

*The appearance of the insulin receptor occurs early enough to be useful for tissue typing.* Fig. 1 displays the time-course relationships between the appearance of an insulin receptor, as assessed by insulin binding to MLC-activated lymphocytes and DNA synthetic rates estimated by uptake of [ $^3$ H]thymidine. In the human MLC studied, DNA synthesis was identical in allogeneic and syngeneic cultures after 1 d of culture, but DNA synthesis in histoincompatible combinations rose on the 4th d of culture, and peaked on the 6th d before rapidly returning to base line. Using the uptake of [ $^3$ H]thymidine, one is unable to determine a positive MLC before the 4th d (Fig. 1). In striking contrast is the time course for the generation of the insulin receptor. By 24 h of culture, significant insulin receptor binding is appreciable in histoincompatible combinations. Insulin binding peaked at day 4 before reaching a plateau through at least the 9th d of study. These studies clearly demonstrate that the insulin receptor generated in human MLC is easily discernable after 24 h of culture, well before the appearance of DNA synthesis.

To strengthen the observation that positive MLC can be identified after 24 h of culture, 30 additional MLC were performed and analyzed at this time (see Table I). In concert with the experience with HLA-identical MLC analyzed at 6 d, the insulin binding was negative in the eight allogeneic, HLA-identical combinations studied at 24 h ( $0.1 \pm 0.1$  pg  $^{125}$ I-insulin bound/ $10^6$  cells). All 22 assays performed on cells from HLA-incompatible MLC were positive at 24 h. Most importantly, the amount of radioreceptor binding correlated with serologic assessment of the HLA com-

patibility. Radioreceptor binding was  $5.3 \pm 0.7$  pg/ $10^6$  cells (range 2.1–6.7) on nylon wool nonadherent responder cells from MLC established between the six combinations totally mismatched for the HLA gene products. Binding was significantly less on cells from 16 cultures exhibiting one-haplotype matches ( $3.2 \pm 0.4$  pg/ $10^6$ , range 1.1–6.1;  $P < 0.02$ ). In seven of eight cultures established between HLA-identical subjects, absolutely no binding was observed. The one culture with trivial binding exhibited only 0.5 pg/ $10^6$  of the radiolabel bound, well below the lower limit of a positive result in our laboratory. The mean result in this group ( $0.1 \pm 0.1$  pg/ $10^6$ ) is not statistically different from zero specific binding. These results confirm that the binding assay can detect with confidence the negative MLC even as early as 24 h. Radiolabel binding in these experiments at 24 h was quantitatively greater than that observed for the time-course experiments (Fig. 1), probably because we employed 2-mercaptoethanol fortified culture medium. Aliquots of those cultures harvested at 6 d revealed no more binding than our initial studies, permitting their inclusion in the 6-d data presented in Table I. The fact that there was no specific binding in the HLA-identical cultures can be taken as proof that the enriched medium with 2-mercaptoethanol alone does not stimulate the formation of insulin receptors on lymphocytes.

## DISCUSSION

We report here a new rapid means by which prospective HLA-D allograft matching may be performed, analysis of the emergence of a T lymphocyte insulin receptor. Advantage is taken of the unique features of the T cell insulin receptor system. Whereas nonactivated lymphocytes lack insulin receptors in the rodent (7) and in man (9, 17), these receptors emerge as a consequence of in vitro and in vivo cellular activation (15, 17). In particular, we have previously shown that the insulin receptor appears on rodent T cells activated in the MLC (18). We confirm that human cells should respond in a similar fashion. Using HLA-typed human cells, we have demonstrated that the appearance of an insulin receptor correlates in every case with a positive intrafamilial MLC identified in a conventional manner by incorporation of [ $^3$ H]thymidine. The insulin-binding assay itself is relatively simple, requires a total of 90 min, uses modest numbers of cells ( $10^7$ ), and is highly reproducible (intratest coefficient of variation, 3%). Generation of lymphocyte insulin receptors requires a mismatch for the HLA-D region between responder and stimulator lymphocytes. In the studies of families with well-defined intra-HLA crossover events, experimental results were consistent with the concept that the emergence of positive radioreceptor binding correlated with a mismatch of that portion of histoincompatible DNA that contained the D allele.

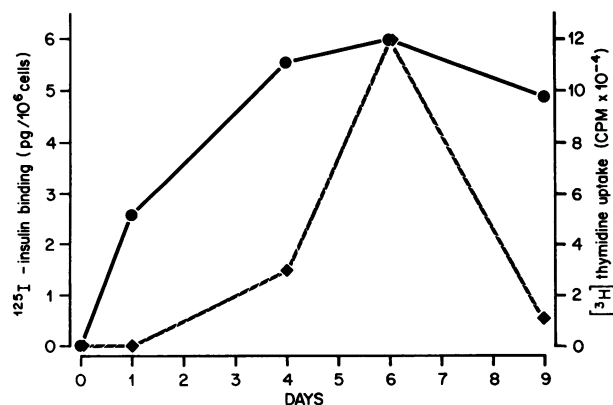


FIGURE 1 Temporal relation between the cellular accumulations of [ $^3$ H]thymidine and the appearance of insulin receptor binding in cells from a reactive human MLC.  $\blacklozenge$ , [ $^3$ H]thymidine uptake;  $\bullet$ ,  $^{125}$ I-insulin binding.

Rapid MLC testing by means of insulin receptor binding may be useful in clinical renal transplantation, as conventional MLC correlate with graft outcome (1, 2). The insulin radioreceptor assay assures that the time constraints for MLC testing imposed upon the transplant team by present preservation techniques can be met. Our data show that the insulin receptor assay becomes positive in 24 h, quantitatively related to the degree of HLA mismatch existent in the given MLC combination. Thus, our studies suggest that an alternative measure of MLC reactivity may be assessed in an attempt to choose donor-recipient pairs with the weakest MLC stimulation, since the data clearly indicate that the radioreceptor assay can quantitatively reflect alloreactivity of MLC-stimulated lymphocytes. Further, failure to generate insulin receptor-positive MLC cells suggests HLA-D identity for the potential donor-recipient pair, a circumstance which seems most favorable for a successful graft outcome (6).

The radioreceptor assay marks a premitotic event in lymphocyte activation. We have shown MLC or mitogen stimulation of responder T cell insulin receptors despite ablation of *de novo* DNA synthesis or cell division (19, 20). Thus, the possibility that the premitotic appearance of the insulin receptor might be a useful tool for clinical transplantation was anticipated.

In summary, we have shown that a new, easy, rapid assay which measures the appearance of T lymphocyte insulin receptor can predict quantitatively the degree of MLC response and can prospectively match the HLA-D gene products. It is hoped that the use of this assay may be the basis of a means to improve outcome from cadaveric renal transplantation. A prospective study correlating the insulin-binding assay at 24 h of MLC with serologic HLA assessment, 6-d thymidine uptake, the clinical renal transplant outcome is in progress.

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