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 [3H]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 crossover 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-hapto-type-mismatched, intrafamilial renal transplants (1–6). Unfortunately, the method by which lymphocyte reactivity in the mixed lymphocyte culture (MLC) 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 (≤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 µg/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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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 10^6 lymphocytes from a responder subject with
10^6 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% CO2 and air. Simultaneously, at
least 10^6 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 x 10^6 cells minimally required for the binding assay to be
performed in duplicate. Elimination of lymphocyte con-
tamination by macrophages is crucial, as macrophages but
not lymphocytes freshly prepared from human blood ex-
press insulin receptors (9). The technique for exclusion of
measurable insulin receptor-bearing residual macrophages
was adequate, as no radiolabeled insulin binding was de-
tected 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 [3H]thymidine and for ins-
ulin 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 Baum 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 μCi of [3H]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 ≥ 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 rela-
tionship 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 ad-
ditional 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, 1.5-10^6 cells from each member of the combination
were placed in 12 x 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% decomplemented normal human
serum, 2 mM glutamine, and 50 μM 2-mercaptopethanol.
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 be-
tween related subjects sharing one HLA haplotype, and 8 be-
tween 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 de-
scribed 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
[3H]-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 Beck-
man 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**
<table>
<thead>
<tr>
<th><strong>MLC Analyzed at 1 and 6 D</strong></th>
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<tbody>
<tr>
<td><strong>No. of haplotype</strong></td>
<td><strong>No. of</strong></td>
</tr>
<tr>
<td><strong>mismatches</strong></td>
<td><strong>cultures</strong></td>
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<td>------------------</td>
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</tr>
<tr>
<td></td>
<td>pg/10^6 cells</td>
</tr>
<tr>
<td>2</td>
<td>5.3±0.7</td>
</tr>
<tr>
<td>1</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>0</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
insulin receptor assay (6.8±1.3 pg/10⁶ cells, range 2.8–9.6) and thymidine assays (SI 45±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±1.0 range 1.6–9.4 pg/10⁶ insulin bound; SI 22±5, range 11–48). The 10 MLC between HLAtwin sibs were negative by both assays (0.1±0.2 pg/10⁶; SI 1.1±0.2). In no case did a single MLC without an HLA mismatch give binding ≥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 [¹⁴C]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 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-

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**TABLE II**

<table>
<thead>
<tr>
<th>HLA Genotypes in Recombinant Families</th>
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<tbody>
<tr>
<td>Family</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>Father</td>
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<tr>
<td>Mother</td>
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<tr>
<td>T</td>
</tr>
<tr>
<td>D</td>
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<td>P</td>
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<td>2</td>
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<tr>
<td>Father</td>
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<td>Mother</td>
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<tr>
<td>B</td>
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<td>D</td>
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<td>P</td>
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<td>3</td>
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<tr>
<td>Father</td>
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<tr>
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<td>B</td>
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<tr>
<td>G</td>
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<tr>
<td>K</td>
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<td>S</td>
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* The underlined alleles represent the observed recombinations.

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**TABLE III**

<table>
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<tr>
<th>Comparison between [¹⁴C]Thymidine Uptake and Insulin Receptor Assay in MLC from Informative Recombinant Pairs</th>
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<tbody>
<tr>
<td>MLC pair</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Family 1</td>
</tr>
<tr>
<td>T plus Pₖ</td>
</tr>
<tr>
<td>D plus Pₖ</td>
</tr>
<tr>
<td>Family 2</td>
</tr>
<tr>
<td>B plus Pₖ</td>
</tr>
<tr>
<td>D plus Pₖ</td>
</tr>
<tr>
<td>Family 3</td>
</tr>
<tr>
<td>K plus Sₖ</td>
</tr>
</tbody>
</table>

SI, stimulation index, calculated as the ratio of the cpm 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 [3H]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 [3H]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±0.1 pg 125I-insulin bound/10⁶ 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 compatibility. Radioreceptor binding was 5.3±0.7 pg/10⁶ 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±0.4 pg/10⁶, 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/10⁶ of the radiolabel bound, well below the lower limit of a positive result in our laboratory. The mean result in this group (0.1±0.1 pg/10⁶) 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 [3H]thymidine. The insulin-binding assay itself is relatively simple, requires a total of 90 min, uses modest numbers of cells (10⁵), and is highly reproducible (intratetst 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](image-url) Temporal relation between the cellular accumulations of [3H]thymidine and the appearance of insulin receptor binding in cells from a reactive human MLC. •, [3H]-thymidine uptake; ○, 125I-insulin binding.

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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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