Third Party Mixed-Leukocyte Culture Test: A Potential New Method of Histocompatibility Testing

(disparity index/graft-versus-host reaction)

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ABSTRACT

The disparity index is an expression of histocompatibility difference between two siblings with identical human leukocyte antigen (HL-A) type who are nonreactive in mixed leukocyte culture (MLC) test. This index is derived from a third-party MLC test. The disparity index was found to be correlated with the severity of graft-versus-host reaction in bone marrow transplantation between HL-A-identical siblings. The disparity index may prove to be a useful means of predicting the severity of graft-versus-host reaction and the outcome of bone marrow graft when HL-A- and MLC-matched sibling donors are studied. The third-party MLC test is offered as a new method of histocompatibility testing. It may provide a useful model for the study of immunogenetics relative to so-called weaker histocompatibility determinants in man and experimental animals.

Since it has been shown in man that lymphocytes carry most (if not all) of the transplantation antigens (1, 2), a proper and precise typing of human lymphocytes would naturally provide a useful means of selecting donor and recipient for the transplantations of tissues and organs in man.

After the demonstration by Dausset (3) of the existence of leukocyte isoantigens in man, more than 30 antigens of the human leukocyte have been identified that belong to the human leukocyte antigen (HL-A) system (4). The extreme polymorphism of the HL-A system makes it difficult to find an HL-A identical donor outside family members.

In the mixed leukocyte culture (MLC) test (5, 6), the sum total of antigenic differences between two individuals is measured regardless of the kind and the numbers of different antigens involved. Genetic studies of the MLC test suggested that a minimum of 20 different alleles is involved (7), and that the same chromosomal region controls both the HL-A system and MLC reaction in man (8). Like the typing of HL-A antigens, it is extremely rare to find MLC-matched pairs outside family members. A nonstimulatory MLC is regularly found in identical twins and in HL-A-identical siblings with rare exceptions (9). Within family members, the prolongation of skin graft and kidney graft are well correlated with the identity of HL-A type of leukocytes and reactivity of leukocytes in MLC tests (10–12). However, these correlations have been generally poor when the donor is outside the family (13, 14). For the practical purpose, at the present time, the best match between the donor and recipient of a transplant is a donor (a) having HL-A identity, (b) having MLC nonreactivity, and (c) deriving from among family members. Even among pairs that fulfill all these three conditions, the graft-versus-host reaction may take place in the recipient of bone marrow transplant, or the grafted organs may gradually lose function unless the donor is an identical twin of the recipient. Thus, it must be assumed that another yet unknown factor(s) must exist that influences the outcome of organ, tissue, and cellular grafts. It is our purpose here to report a new method of histocompatibility testing that we believe will further discriminate the degree of incompatibility in HL-A identical, MLC nonreactive sibling pairs.

MATERIALS AND METHODS

Mixed leukocyte culture was performed according to the methods of Sengar et al. (15) with minor modifications. Briefly, the leukocytes (95% or more were mononuclear cells) were isolated according to the method of Thorby and Breatle (16). The culture media consisted of RPMI-1640 (Associated Biomedies, Inc., Buffalo, N.Y.) containing 20% pooled normal human AB serum. Each culture tube (12 × 75 mm, Falcon Plastic, Oxnard, Calif.) contained 5 × 10⁴ responding cells and an equal number of mitomycin-treated stimulating cells in 0.2 ml of culture media. The tubes were incubated at 37°C in an incubator with 7.5% CO₂ and humidity for 4 days. At the end of this incubation, 0.5 μCi of [methyl-3H]dT (2.0 Ci/mol, New England Nuclear Corp., Boston, Mass.) in 50 μl of tissue culture medium (RPMI-1640) was added to each tube. The tubes were incubated for another 24 hr under the same conditions. At the end of the second incubation ([3H]dT pulse), the entire culture mixture was then filtered through a Millipore filter (0.4 μm pore size, 13 mm in diameter, Millipore Corp., Boston, Mass.) with the apparatus described (17). The filter paper with entrapped leukocytes was washed successively by filtration of 4 ml of cold physiologic saline and 4 ml of 5% trichloroacetic acid. Additional suction was applied for 10–15 sec for the removal of moisture from the filter paper. The filter paper was carefully transferred with forceps to the bottom of a glass specimen vial (15 × 45 mm, Kimble Opticlear produced by Owen-Illinois Co., Toledo, Ohio) and 0.1 ml of 0.2 N KOH was added to dissolve the collected precipitate from the filter paper. The tube was placed in a standard scintillation vial. 30 min later, 3 ml of scintillation solution (17) was added to the specimen vial; after 2 hr, the radioactivity of tritium was measured in a liquid scintillation counter, Beckman model LS 250. The degree of incorporation of [3H]dT
into the DNA of leukocytes is proportional to the degree of lymphocyte transformation in the MLC test, and is expressed as cpm per 5 \times 10^4 leukocytes. The third-party MLC test was performed on the leukocytes of siblings with HL-A-identical genotype and nonreactive MLC test. The cells of the siblings were treated with mitomycin and incubated with the leukocytes of a third-party unrelated individual in the standard one-way MLC test described above.

The disparity index (DI) was derived from the results of third-party MLC test according to the formula below:

\[
DI = \frac{|X_{Am} - X_{Bm}|}{\sqrt{X_{Am} + X_{Bm}}}
\]

where \(X_{Am}\) and \(X_{Bm}\) represent mitomycin-treated stimulating leukocytes from an HL-A identical, MLC nonreactive sibling pair, A and B, respectively; \(X\) represents responding leukocytes from an unrelated individual; and \(X_{Am}\) and \(X_{Bm}\) represent the degree of response of leukocytes from X (unrelated individual) to \(Am\) or \(Bm\), respectively, expressed as cpm.

**RESULTS**

An example of calculation of the disparity index from the results of a third-party MLC test is shown in Table 1, i.e., \(X_{Am} = 1276, X_{Bm} = 1110\). Therefore, \(DI = 0.14\). Table 2 shows the results of a third-party MLC test in three HL-A identical siblings \((A, B, \text{and } C)\) in a family with two unrelated individuals \((X_1, X_2)\). The disparity indices between \(AB, BC, \text{and } AC\) are 0.63, 0.62, and 0.01 for \(X_1\) and 0.63, 0.62, and 0.03 for \(X_2\), respectively. There is a similarity between the disparity indices for \(X_1\) and those for \(X_2\).

Table 3 shows the results of a third-party MLC test in identical twins. The capacity of leukocytes of twins to stimulate the third-party leukocytes \((X_1 \text{ and } X_2)\) were close to identity, resulting in disparity indices near zero.

Table 4 summarizes results of third-party MLC tests on HL-A identical siblings from 12 families. The disparity index ranged from 0.01 to 0.79. Four sibling pairs \((\text{no. } (1) \text{ Pe, } (2) \text{ By, (6) Fe, (8) Tu families})\) were the HL-A- and MLC-identical donors and recipients of bone marrow transplantation for reconstitution of combined immunodeficiency disease. The severity of the graft-versus-host reaction appeared to be correlated well with the disparity index between the donor and recipient, i.e., the smaller the index, the lesser the graft-versus-host reaction.

**DISCUSSION**

The importance of pursuing improved histocompatibility testing has been strongly affirmed since the individual specific tissue isoantgens (histocompatibility antigens) are under dominant genetic control. These histocompatibility antigens are mainly responsible for provoking the complex events involved in rejection of grafted tissues. It seems from both clinical and experimental study that lesser immunogenetic differences between a donor and recipient can contribute to better prognosis of the fate of grafted tissue.

Currently, available methods of testing histocompatibility can be divided into two categories: (a) the histocompatibility typing and (b) histocompatibility matching. The term "typing" is used for techniques that provide identification of
individual antigen(s) that can be recognized specifically by serological means. The serotyping of human leukocytes has provided a major advance and has led to the discovery and definition of the complex HL-A system in man (4). Matching methods, however, estimate the sum of the several independent influences of transplantation antigens on the reactions between donor and recipient.

A number of matching methods have been used in man to select suitable donors for transplantation. The third man test (18, 19) compares the accelerated rejection of second skin grafts by the indifferent recipient, a phenomenon clearly dependent upon antigens that are shared by the donor of the initial graft (the intended recipient) and the potential donor. The normal lymphocyte transfer (NLT) test (20, 21) measures the skin reaction (delayed type) of potential donor upon intradermal injection of leukocytes from a potential recipient. The lesser the degree of the reaction, the longer is the predicted survival of the graft. The skin reaction is believed to be due to a local graft-versus-host reaction elicited by the injected immunocompetent lymphocytes. The major disadvantages of both methods are the potential hazard of transmitting infectious agents to the test subject and the qualitative nature of the assay. The one-way MLC test by either irradiated or mitomycin-treated cells as a stimulator (22, 23) measures the unidirectional reaction of one individual's cells against allogeneic leukocytes in vitro. Since this test is performed in vitro, the danger of cross infection is eliminated and the test is quantitative. The ready access to leukocytes provides a further advantage of MLC testing over the other two matching methods. The over-all correlation between the MLC test, the survival of skin and kidney graft, and the HL-A typing have been shown generally to be good among the members of the same family. The correlation is generally poor between unrelated individuals. At present, the best possible match, besides identical twins, is siblings with identical HL-A type and a non-reactive MLC test.

The third-party MLC test described herein, we contend, provides an additional method of histocompatibility matching that will further discriminate antigenic disparity between siblings with identical HL-A type and nonreactive MLC test. In the third-party MLC test, the stimulating capacity (hence the antigenicity) of leukocytes from two siblings against the leukocytes of the same unrelated individual is measured. Antigenic differences between the leukocytes of two donors are thus reflected in the differences of stimulation of the leukocytes of the third party. The third-party MLC test is similar to the third-man test in a sense that the histocompatibility antigen(s) of both prospective donor and recipient are tested upon a third-party individual. However, in contrast to the third-man test, a prior sensitization of the third individual is not required for this analysis. Further, in the third-man test the similarity between the donor and the recipient is sought in the accelerated rejection of skin graft, while in the third-party MLC test more direct measure of the genetic disparity between the donor and recipient is provided.

Neither the normal lymphocyte transfer test nor the third-party MLC test requires presensitization. The two methods differ in that the responding cells in the third party MLC test are the lymphocytes of the third party, whereas in the normal lymphocyte transfer test, the responding cells must be the immunocompetent lymphocytes from the prospective recipient of the organ that provokes the reaction in the skin of the prospective donor. For two reasons, the normal lymphocyte transfer test is not suitable for the matching of donor and recipient for bone marrow transplantation: (a) the hazard of the transplant relates to the graft-versus-host reaction produced by donor cells where the recipient is often immunoincompetent and (b) in present marrow transplantations, the recipient regularly is lacking the thymus-derived immunocompetent lymphocyte that elicits delayed type skin reaction in the test subject.

At present, the third-party MLC test is applicable mainly in siblings with identical HL-A type, whose leukocytes are non-reactive in the MLC test. In these conditions, it is assumed that the major histocompatibility antigens are identical. Therefore, the third-party MLC test and the disparity index derived therefrom probably reveal differences in minor histocompatibility antigens. Nonetheless, this method of histocompatibility matching may provide a new approach to the analysis of immunogenetics in man and experimental animals.

Our preliminary studies of the disparity index and severity of graft-versus-host reaction in patients after bone marrow transplantation suggest that the disparity index between the donor and the recipient may have predictive value on the severity of graft-versus-host reaction in the recipient. Since the graft-versus-host reaction is the single most important limiting factor in successful marrow transplantation, the disparity index should contribute a useful means of selecting donors, calculating the number of marrow cells that can be safely transplanted, and thus minimize graft-versus-host reactions. We would predict that the third-party MLC test will provide a useful additional means of selecting donors among both related and unrelated individuals.

The results of third-party MLC test in identical twins indicate that the disparity index is influenced by genetically controlled factor(s). The major factors controlling the disparity index could be minor histocompatibility antigens or differences controlled by the recently described "MLC loci" or "hyper-sensitivity delayed reaction (HDR) locus" (24). That the severity of the graft-versus-host reaction was found to be related to the disparity index between donor and recipient leads us to postulate that a separate locus controlling the disparity index may exist. This locus, however, could be the same or could be closely related to the "HDR locus." Further studies to analyze the genetic control of the third-party MLC test in man and experimental animals should be performed.

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