Utilization of Cultured Human Lymphoid Cells for Detection of Humoral Sensitization in Prospective Recipients of Kidney Transplants

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ABSTRACT Prospective recipients of kidney transplants were tested for lymphocytotoxicity; from these we selected 102 sera that lacked cytotoxic antibodies against peripheral lymphocytes from at least 80 unrelated subjects. To detect humoral sensitization, we then reacted these with 17 cultured human lymphoid cell lines having different HL-A phenotypes. Cytotoxic antibodies reacting with these cultured cells were now detected in some of the sera. These antibodies were not directed against HL-A antigens, yet mediated lysis of target cells in the presence of rabbit but not of human or guinea pig complement. Furthermore, they activated the classical pathway of the rabbit complement system. Later, a significant association was found between occurrence of cytotoxic antibodies and rejection of the transplant. Thus, cultured human lymphoid cells, because of their great susceptibility to complement-mediated lysis, appear to be useful in detecting humoral sensitization in candidates for kidney grafts.

INTRODUCTION

A compelling body of evidence indicates that the presence of lymphocytotoxic antibodies in the sera of prospective recipients of kidney transplants subsequently affects graft survival unfavorably (1). Sometimes these antibodies can be detected only when the sensitivity of the lymphocytotoxic test is increased by prolonging the incubation time, adding sublytic amounts of rabbit anti-human lymphocyte serum to the reaction mixture, or using lymphocytes treated with trypsin as target cells (2). There remains, however, a certain percentage of patients who reject their kidney transplants acutely, even though no cytotoxic antibodies to peripheral lymphocytes are detected by any of the above means. In an attempt to determine whether these patients do indeed have cytotoxic antibodies that play a role in the rejection of the grafts, we have screened a number of sera from prospective recipients of kidney transplants against a panel of cultured human lymphoid cells and correlated the results with graft survival.

Cultured human lymphoid cells are more sensitive than peripheral lymphocytes to HL-A antibody-mediated lysis because the former elicit higher titers of HL-A alloantisera (3, 4) and are lysed by human complement activated by HL-A antibodies (5); human serum, though, is a poor source of complement when peripheral lymphocytes are the targets (5). Although cultured lymphoid cells have more antigenic determinants than peripheral lymphocytes (6, 7), the reason for their greater sensitivity is not completely clear. Cultured cells may also have determinants not found on peripheral lymphocytes (3, 8–11). No data are available on the sensitivities of membranes from cultured cells and peripheral lymphocytes to the lytic action of complement. The data presented in this paper indicate that cultured lymphoid cells can be useful in detecting humoral sensitization in patients selected because their sera do not react with peripheral lymphocytes. Furthermore, the antibodies thus detected are directed against determinants other than HL-A antigens, cytolytic in conjunction with rabbit complement but not with human and guinea pig comple-

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ment, and activators of the classical pathway of the complement system.

METHODS

Cultured human lymphoid cells. 17 cultured cell lines, WI-L2, NC37, RPMI 1301, RPMI 1788, RPMI 4098, RPMI 6237, RPMI 6410, RPMI 8866, RAJI, Daudi, UM 1, UM 43, UM 54, UM 56, SCRF 5006, and NC 37 and RAJI resistant to 5-bromodeoxyuridine (NC 37-BU and RAJI-BU),[1] were used in this study. The cell lines RPMI 1788, RPMI 4098, RPMI 6410, RPMI 8866, RAJI, and RAJI-BU were grown in suspension in medium RPMI 1640 containing 10% fetal calf serum. The remaining cell lines were perpetuated in minimum essential medium (MEM) containing 10% fetal calf serum. All the culture flasks except those containing Daudi cells were rotated continuously on a platform shaker. Cell viability was assessed by the uptake of trypan blue. To avoid variability in cell surface expression of antigens during different phases of the growth cycle, only cells in log phase were used. All cells were washed once with Hanks' balanced salt solution (BSS), resuspended in BSS, and adjusted to a concentration of 2×10^6 cells/ml.

Human peripheral lymphocytes. These cells were obtained from heparinized blood by the Ficoll-Isopaque method (12).

Platelet suspensions. Suspensions of platelets from 70 donors representing all known HL-A specificities were adjusted to a concentration of 1×10^9 cells/ml saline containing 0.1% NaN_3. Platelet suspensions were stored at 4°C for periods up to 1 yr.

Soluble antigens utilized in the inhibition test. Soluble serum HL-A antigens were partially purified by ion exchange chromatography (13, 14) and gel filtration[2] from sera of five donors bearing HL-A 1, 2, 9, 5, 7, and 12 specificities in different combinations. Membrane lymphocyte antigens were solubilized from cultured human lymphoid cells RPMI 1788, RPMI 4098, and WI-L2 by hypertonic salt extraction (15). Partially purified serum HL-A antigens and membrane lymphocyte antigens were able to specifically inhibit cytotoxic HL-A antisera. β_2-microglobulin, a generous gift from Dr. M. D. Poulik (Detroit, Mich.) was purified from urine according to the method of Berggard and Bearn (16). The protein was judged to be pure by starch gel, immunoelectrophoresis, and ultracentrifuge criteria.

Sera. Sera were obtained from 115 pregnant women and 102 patients who subsequently received kidney transplants. 45 of the grafts were successful for at least 6 mo; the remaining 57 failed within 3 mo. Sera from transplant recipients were selected in P. I. Terasaki's laboratory on the basis of containing no cytotoxic antibodies reacting with peripheral lymphocytes from at least 80 unrelated people. These sera were coded and shipped to our laboratory at Scripps Clinic and Research Foundation. The sera were stored at −20°C for periods up to 2 yr and were frozen and thawed several times.

Complement. Human complement was from the pooled sera of five donors who had no previous history of pregnancy or blood transfusion. Guinea pig complement was from the pooled sera of 12 randomly selected guinea pigs. Rabbit complement was from the pooled sera of nine rabbits selected after individual bleedings proved nontoxic in the cytotoxic assay with peripheral lymphocytes from four subjects. Rabbit complement (5 vol) was absorbed with 1 vol of packed, washed, cultured human lymphoid cells by incubation at 4°C for 60 min with mixing at 10-min intervals. To reduce loss of complement activity, the absorption of rabbit serum was performed in the absence of free divalent cations (5). Complement was divided into small aliquots (300 μl), stored at −70°C, and used only once after thawing.

Cytotoxic test. Unless otherwise stated, the eosin exclusion test was used with both cultured human lymphoid cells (17) and peripheral lymphocytes (18). Cold lymphocytotoxic antibodies were tested for by performing the test

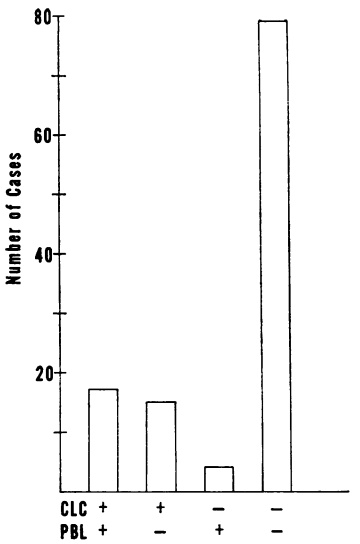

FIGURE 1 Reactivity of sera from pregnant women with a panel of 10 cultured human lymphoid cells (CLC) and with peripheral lymphocytes (PBL) from at least 90 unrelated subjects.

TABLE I

Distribution of 32 Cytotoxic Sera from Pregnant Women According to the Number of Lymphoid Cell Lines Giving Positive Reactions

Number of cell lines reacting	Number of positive sera
1	8
2	5
3	6
4	1
5	5
6	2
7	0
8	2
9	2
10	1

[1] *Abbreviations used in this paper:* ALS, antihuman lymphocyte serum; BSS, balanced salt solution; BU, resistant to 5-bromodeoxyuridine; EGTA, ethylene-glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; MEM, minimum essential medium.

[2] Unpublished results.

TABLE II

Reactivity of the Cytotoxic Sera from Prospective Recipients of Kidney

Serum number	Cell lines and HL-A phenotypes*					
	RPMI 1788 (2, 10, 7, 14)	RPMI 4098 (3, W29)	RPMI 6410 (2, 7, 12)	RPMI 8866 (2, 3, 7, 12)	WI-L 2 (1, 2, 5, 17)	RAJI (3)
Individuals who later rejected renal grafts.						
27072	+	−	−	−	−	−
27844	−	−	−	−	−	−
28160	+	+	+	−	+	+
29810	−	+	+	+	−	−
32140	+	−	−	−	+	+
33662	+	−	−	−	−	−
35080	−	+	−	−	−	−
47104	ND	−	+	−	−	−
51357	ND	−	−	−	−	−
52190	ND	−	−	−	−	+
58978	ND	−	−	−	−	+
61070	ND	−	+	−	−	−
62718	ND	−	−	−	−	−
63402	ND	−	−	−	−	+
Individuals who later accepted renal grafts for 6 mo or longer.						
47895	−	−	+	−	−	−
51392	−	+	+	+	−	+
52609	−	−	+	−	−	−

* Other cell lines shown in Methods were not reactive with these sera.

‡ ND, not done.

at 15°C (19). The cytotoxic test with the addition of sublytic amounts of antihuman lymphocyte serum and with prolonged incubation time was performed as described (2). The tests were read without knowledge of the outcome of transplantation.

Inhibition of cytotoxic test by soluble antigens and platelet suspensions. This procedure was done as previously described (20). Briefly, 1 μl of a platelet suspension or of soluble antigens was added to 1 μl of serum at twice the concentration required to lyse 95% of target cells. After 60 min of incubation at room temperature, cultured human lymphoid cells were added, and all subsequent steps were the same as in the cytotoxic test.

Chelating agent. To chelate calcium, neutralized EGTA (ethylene-glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetra-acetic acid, Calbiochem, San Diego, Calif.) was added to the complement to a final concentration of 0.01 M.

Statistical analysis. The results of screening the sera against the panel of peripheral lymphocytes were analyzed by Boolean factor analysis[3] and by correlation coefficients.

RESULTS

In a preliminary study we compared the sensitivities of cultured human lymphoid cells and of peripheral lymphocytes as targets in the cytotoxic test. To this end, 115 sera from pregant women were screened against 10 cultured human cell lines and peripheral lymphocytes from at least 90 unrelated subjects. The number of positive reactions against cultured human lymphoid cells was significantly higher than against peripheral lymphocytes ($\chi^2 = 32.9$, $P < 0.0001$). (Fig. 1) Distribution of the sera according to the number of cultured human lymphoid cell lines with which they reacted is depicted in Table I. The reactivity of the sera with peripheral lymphocytes in terms of percentage of positive reactions and their strength indices (% positive reactions with total killing of target cells) did not correlate with the number of cultured human lymphoid cell lines yielding positive results.

Of the four sera positive only with peripheral lymphocytes, three contained antibodies against HL-A specificities 17, 5, and 12 (all of which were represented on the panel of cultured human lymphoid cells), and the fourth contained antibodies that did not correlate with any known HL-A specificity.

Of the sera from candidates for allografts, 14 of 57 sera from patients who subsequently rejected kidney transplants contained cytotoxic antibodies against cultured human lymphoid cells preoperatively. Only 3 out of 45 patients with successful grafts had prior cytotoxic antibody; utilizing the Fisher correct test, we found a significant association between the occurrence of cytotoxic antibodies and the outcome of the transplant ($P < 0.05$).

The pattern of reactivity of the sera against the panel

[3] Mickey, M. R. Unpublished results.

*Transplant with the Panel of Cultured Human Lymphoid Cells**

Cell lines and HL-A phenotypes*							
NC 37-BU (ND‡)	NC 37 (ND)	1301 (ND)	UM 1 (2, 9, 5)	UM 43 (ND)	UM 54 (1, 2, 5, 7)	UM 56 (3, 5, 7)	UM 61 (1, 2, 17, W5)
ND	ND	ND	−	−	−	−	−
ND	ND	ND	−	+	+	−	+
ND	ND	ND	−	+	−	−	−
ND	ND	ND	−	−	−	+	−
ND	ND	ND	+	+	+	−	+
ND	ND	ND	−	−	−	−	−
ND	ND	ND	−	−	−	−	+
+	−	+	−	ND	−	−	−
+	−	+	−	ND	−	−	−
+	−	+	−	ND	−	−	−
+	+	+	−	ND	−	−	−
+	−	+	−	ND	−	−	−
+	+	+	−	ND	ND	ND	ND
−	+	+	ND	ND	ND	ND	ND
−	−	−	ND	ND	ND	ND	ND
−	−	−	−	−	+	+	−
+	−	+	−	ND	−	−	−

of cultured cells is reported in Table II. These results, together with the inability of partially purified serum HL-A antigens and of platelet suspensions from a panel of 70 donors having all known HL-A specificities to inhibit the activity of cytotoxic antibodies against cultured human lymphoid cells, exclude any clearly defined HL-A specificity of these cytotoxic antibodies. Purified β_2-microglobulin also could not inhibit these cytotoxic antibodies that, however, were inhibited by antigens solubilized from cultured lymphoid cells that reacted with these antibodies in the cytotoxic test (Fig. 2). In addition, no relationship was observed between the reactivity of cultured human lymphoid cells and presence of β_2-microglobulin on the cell surface, type of immunoglobulins secreted, type of C3 receptors, or source of the cells. The titers of cytotoxic antibodies present in transplant recipients' sera varied from cell line to cell line between 1:8 and 1:256.

To investigate whether the cytotoxic antibodies detected in prospective recipients of kidney transplant were directed against Epstein-Barr virus-related antigens, cultured human lymphoid cells in which the expression of these antigens was induced by treatment with 5-bromodeoxyuridine were utilized as targets in the cytotoxic test. Cells RAJI-BU were not reactive with sera positive to the parental cell line, while cells NC37-BU acquired the reactivity with sera negative to the parental cell line.

To determine the requirements of these cytotoxic antibodies for complement, their cytotolytic potential was determined by reacting them with either rabbit, guinea pig, or human complement (Fig. 3). In this regard, these antibodies resembled HL-A antibodies because rabbit serum was the most effective source of complement. It has recently become apparent that activation of complement

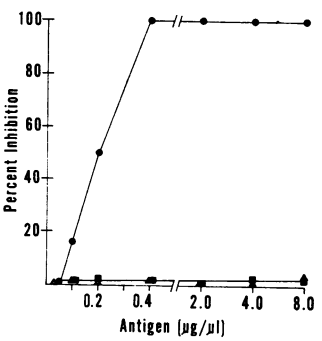

FIGURE 2 Ability of partially purified HL-A antigen from serum S. G. (HL-A 2,9,5) (△—△), β_2 microglobulin (■—■), and antigens solubilized from cultured human lymphoid cells RPMI 6410 (●—●) to inhibit the cytotoxic activity of serum 28160 towards the cultured human lymphoid cell line WI-L2.

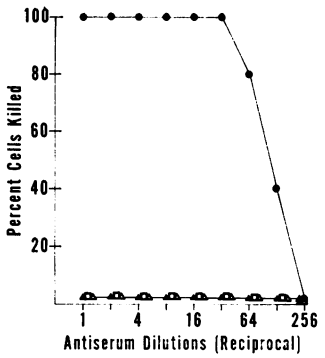

FIGURE 3 Cytolytic potential of serum 35080 in conjunction with human complement (▲—▲), guinea pig complement (△—△), rabbit complement (●—●), or rabbit complement in which activity of the classical pathway has been inhibited by EGTA (final concentration 10 mM) (□—□). Cultured human lymphoid cells RPMI 4098 were the targets in this microcytotoxic test.

can be achieved through two pathways (21–23): one, the classical pathway, utilizes all nine known complement components and requires both calcium and magnesium ions. The other, the alternate pathway, activates complement at the level of C3 and uses the late-acting components, while by passing C1, 4, and 2, requiring magnesium but not calcium (24). All the antibodies used in this study activated the classical pathway of the complement system, as lysis of target cells was prevented by treatment of complement with EGTA at a final concentration of 10 mM (Fig. 3); under our experimental conditions EGTA depleted the reaction mixture of calcium but not of magnesium.

Since previous work with peripheral lymphocytes had shown that prolongation of incubation time increased the sensitivity of the lymphocytotoxic test, we prolonged the incubation time from 2 to 4 h and tested sera from all prospective recipients against the panel of cultured lymphoid cells. The frequency of positive reactions increased but did not correlate with the outcome of transplants. Also, the determinations of cytotoxic antibodies reacting with cultured human lymphoid cells in the cold did not correlate with the results of the transplants.

27 of the sera from patients who later rejected the transplants were tested against cultured human lymphoid cells and peripheral lymphocytes from 50 unrelated subjects; in the latter group, either the reaction was boosted by the addition of sublytic amounts of rabbit antihuman lymphocyte serum (ALS test) or the incubation time was prolonged to 4 h (long test). As shown in Table III, of these 27 sera, 5 reacted with cultured lymphoid cells in a standard cytotoxic test, 7 with peripheral lymphocytes in the ALS test, and 2 in the long test. Considering the sera individually, three reacted with cultured lymphoid cells but not with peripheral lymphocytes, two reacted with cultured lymphoid cells and in the ALS test, two did not react with cultured lymphoid cells but reacted both in the ALS and in the long test and, finally, one serum reacted only in the ALS test.

TABLE III

Reactivity of Cultured Human Cell Lines and Peripheral Lymphocytes with Sera from Prospective Recipients whose Subsequent Kidney Grafts Failed

	Cultured cell lines, standard lymphocytotoxic test	Peripheral lymphocytes*	
		ALS‡	Long incubation§
+sera	5	7	2
−sera	22	20	25

* Cells from 50 unrelated subjects.

‡ The lymphocytotoxic test was boosted by the addition of sublytic amounts of rabbit ALS.

§ The incubation time of the lymphocytotoxic test was prolonged from 2 to 4 h.

DISCUSSION

The present investigation has shown that cultured human lymphoid cells can be used to detect humoral sensitization in some prospective recipients of kidney transplants. 24% of recipients who rejected the kidney transplants had prior cytotoxic antibodies that reacted with cultured human lymphoid cells, but not with peripheral human lymphocytes from at least 80 unrelated individuals. Studies in progress by Drs. Ting and Terasaki indicate that some of these sera can trigger the killing of target cells in the antibody-mediated lympholysis test. Some sera that were positive to cultured human lymphoid cells did not react with peripheral lymphocytes from 50 unrelated donors, even when the sensitivity of the cytotoxic test was increased by either prolonging the incubation time or by adding sublytic amounts of ALS to the reaction mixture. These data suggest that the antibodies may be directed to antigens not expressed on peripheral lymphocytes, or antigens may be present in so low a density that they cannot activate the complement system after combining with antibodies.

The cytotoxic antibodies detected preoperatively in subsequent recipients of kidney transplants were not directed against HL-A antigens, because reactivity of the sera with cultured lymphoid cells did not correlate with their HL-A antigenic profile. Furthermore, the cytotoxicity of the sera could not be inhibited by platelets from a panel of donors bearing all the known HL-A specificities. The pattern of reactivity of the recipients' sera with the panel of cultured lymphoid cells suggests that cytotoxic antibodies display various specificities and that the corresponding antigens detected are qualitatively dif-

ferent. The nature of these antigenic receptors is not clear at the present time, although they do not appear to be related to the presence of β_2-microglobulin on the cell surface, the type of immunoglobulin secreted, the type of C3 receptors, or the source of the cells. The possibility that they may be membrane antigens related to the Epstein-Barr virus must be considered, since some sera did not react with the cell line NC37 but did react with the sublime, in which the expression of Epstein-Barr virus-related membrane antigens was induced by 5-bromodeoxyuridine (25).

Antibodies detected in future transplant recipients can mediate lysis of lymphoid cells only in conjunction with rabbit complement and through the activation of the classical pathway of the complement system, as lysis was prevented by the selective binding of calcium. In this property these antibodies are similar to HL-A alloantibodies, which are able to activate the classical, the alternate, or both pathways of the human complement system (26), but which can only activate the classical pathway of rabbit complement (27). Since neither the antibodies of patients who rejected their transplants by immunologic mechanisms nor the antibodies of recipients with successful grafts were able to activate human complement in vitro, it was impossible to determine if there was any difference in the pathway of complement activation that might explain the different outcomes of the transplants.

The experimental data recently reported by Morris and Dumble (28) and those obtained in this study indicate that screening sera from prospective recipients of transplants with a panel of cultured human lymphoid cells can improve the selection of patients and reduce the risk of graft rejection. Even if other investigators confirm these data in a large series of patients, two reservations should be kept in mind when considering the possible biological roles of these antibodies in the pathogenic mechanism of kidney transplant rejection: first, these antibodies cannot mediate lysis of human lymphoid cells in conjuction with human complement but require the concomitant presence of natural antibodies against human lymphoid cells present in rabbit complement (5); second, in the reactivity requirement of complement and activation of the complement system, no difference exists between cytotoxic antibodies detected in sera from patients who rejected the graft by immunological mechanisms and from patients who had favorable graft outcomes. Unfortunately, the limited amounts of available sera kept us from determining the immunoglobulin class of these antibodies.

From the practical standpoint some considerations seem to be warranted: previous studies (29) have shown that some cultured human lymphoid cells change in their susceptibility as they move through the growth cycle. Therefore, cells used as targets in the cytotoxic test should be in a given stage of their growth cycle, thus making it possible to compare results obtained in different experiments or in different laboratories. Furthermore, the data reported in Table II indicate that the antigenic system against which cytotoxic antibodies detected in prospective transplant recipients are directed is polymorphic. Characterization of the antigenic specificities would lead to selecting the best cultured human lymphoid cell lines to be used as targets. All of the above will improve the efficiency of detecting humoral sensitization in prospective transplant recipients and, combined with tests measuring non-complement-fixing antibodies and cellular immunity, will increase the chances of successful kidney grafts.

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