RELATIONSHIP OF MIXED LYMPHOCYTE CULTURE RESPONSE TO HL-A HISTOCOMPATIBILITY ANTIGENS

EFFECT OF ALLELE PLUS ONE ANTIGEN MATCH*

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It is now well established that compatibility for the *HL-A* antigens between donor and host siblings is generally associated with prolonged excellent function of renal allografts (1–6). Although it may not be possible to identify all of the possible antigens of both segregating series in transplant pairs, nonreactive mixed lymphocyte cultures (MLC)[1] have proven to be excellent predictors of *HL-A* compatibility and graft function.

Controversy remains, however, as to what criteria of compatibility determined either by serologic typing (1–6) for *HL-A* antigens or degree of stimulation in MLC (1, 2, 7–13) can be relied upon to predict the next level of comparatively good transplant success. In this regard, Bach and others (2, 14–16) have stressed that within families donor-host compatibility for one allele is usually associated with intermediate stimulation in MLC as compared with two-allele compatibility or disparity. Seigler et al. (7), however, have noted rather remarkable overlap between the MLC responses between one- and two-allele mismatched cell donors.

We have noted the same variability, but now report that a subgroup with low MLC stimulation may be distinguished by the fact that the donor and host pair share one allele plus one antigen of the second unmatched allele as determined by lymphocytotoxicity testing for *HL-A* antigens.

Materials and Methods

16 families with at least four members were included in which genotyping for two segregating series of the *HL-A* locus was established on the basis of positive identity of at least three and sometimes four antigens. Serologic typing and MLC tests were all performed as a part of the work-up of one family member as a potential renal transplant recipient. The potential

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[1] *Abbreviations used in this paper: F*, stimulation index; G, greater response; I, intermediate stimulation; IN, incompatibility number; MLC, mixed lymphocyte cultures; PHA, phytohemagglutinin; R, responding cells; Rm, mitomycin-treated responding cells; Sm, mitomycin-treated stimulating cells.

recipient was not on immunosuppressive drugs and azotemia was usually controlled by hemodialysis.

As a separate part of the study two additional families were studied. In one instance, family I-50, the father and mother shared the allele *HL-A 1 + 8* and *HL-A* compatibility existed by serologic criteria between the father and two siblings, one of whom became the recipient of one of the father's kidneys. The second large healthy family, I-75, composed of 16 siblings, provided the opportunity to investigate the response between pairs of all possible alleles.

In the serologic studies a panel of 68 antisera detecting *HL-A* antigens *1, 2, 3, 9, 10, 11, W-27, W-28, 5, 7, 8, 12, 13, W-5, W-10, W-15, W-17, W-19* was employed in a slight modification of a two-stage lymphocytotoxicity test described by Amos et al. (17). Blood was collected in heparin and sedimented with methyl cellulose. Residual erythrocytes were removed by lysis with an 0.8% ammonium chloride–0.1% ethylenediaminetetraacetate (EDTA) solution, pH 7. After passage through nylon, the lymphocytes were sensitized at 25°C for 30 min, washed with barbitone buffer, and incubated with 5 μl of fresh rabbit complement at 37°C for 60 min. The cells were washed with 5 μl of 2% EDTA (pH 7)–1.3% NaCl before and after staining with freshly diluted 0.3% trypan blue in 2% EDTA–1.3% NaCl. All sera were tested in triplicate. Complete typing was frequently performed on more than one occasion. Serologic typing was also performed by Dr. Paul Terasaki on a few of the families.

One-way MLC were prepared by a modification of the method described by Bach and Voynov (18) employing 3×10^5 responding cells (R) and 7×10^5 stimulating mitomycin-treated cells (Sm) in 2.0 ml of culture media. In the earlier cultures 20% autologous plasma was included, but later cultures were prepared in 20% pooled human plasma shown to give an equivalent response in comparative tests. Stimulating cells were treated with mitomycin C, 125 μg/10–50 $\times 10^6$ cells (Nutritional Biochemicals Corp., Cleveland, Ohio) and washed before addition to the cultures. Triplicate cultures of all pairs (e.g. R + Sm) were compared to sextuplicate cultures of control responding cells incubated with mitomycin-treated cells (R + Rm). As an additional check on the capacity of the cells to respond, 20 μl of phytohemagglutinin (PHA, Burroughs Welcome & Co., Inc., Tuckahoe, N.Y.) was investigated in triplicate, i.e., R + Rm + PHA. Mixed cultures were incubated for 7 days at 37°C in an atmosphere of 4% CO_2, and PHA-stimulated cultures for 3 days. After incubation, 2.0 μCi tritiated thymidine (in 50 μl volume) was added to each culture for 4 hr after which the reaction was stopped by centrifugation and freezing the cell pellet at −20°C. Nucleoproteins were precipitated with trichloroacetic acid, washed, solubilized, and placed in a vial containing 6% Liquifluor (New England Nuclear, Boston, Mass.) in toluene. Incorporation of radioactivity was determined in a Packard Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Ill.) and the activity expressed in counts per minute after correction for background. Unless otherwise indicated the stimulating index (F) of each set of triplicate mixed cultures for each responding cell was expressed as a ratio of the sextuplet controls, i.e., R + Sm/R + Rm. Statistical analysis of the data was performed using the Student's t test.

RESULTS

As a guide to a cell's capability to respond, we chose PHA-stimulated cultures as a nonspecific indicator. If the PHA response fell significantly below the observed range for that set of experiments, all data related to that cell type were discarded. In fact, this was observed on only five occasions and in every case evidence of hyporeactivity was also discernible from the mixed culture results. When the maternal response to all children was low in comparison with the father and/or *HL-A*–incompatible siblings, those data were excluded.

After these deletions, 214 mixed cultures were available for comparative analysis with lymphocytotoxicity *HL-A* antigenic typing. The comparisons

were analyzed in three ways: (*a*) on the basis of an antigen match-grade system utilized by Dr. Paul Terasaki, (*b*) on the basis of allelic compatibility, and (*c*) on the basis of a modification of these two approaches that will be discussed below.

Comparison of MLC with antigen match-grade is demonstrated in Fig. 1 and Table I. In this analysis match-grade *A* indicates complete identity of all detectable donor and host *HL-A* antigens; *B*, *HL-A* incompatibility without

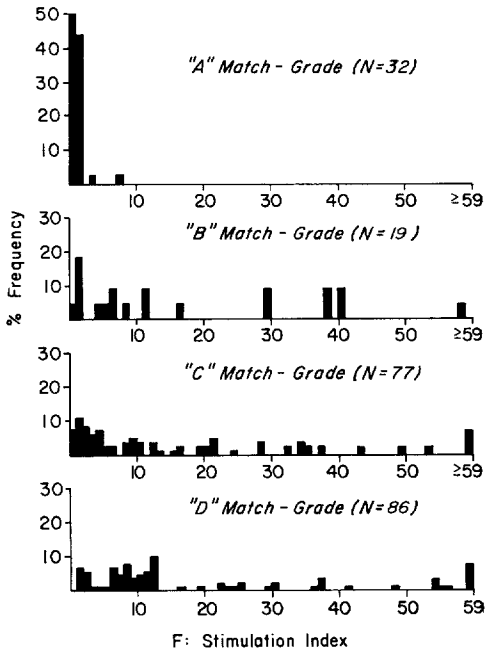

FIG. 1. Relationship of MLC response to tissue match-grade in 214 cultures performed within 16 families. Tissue match-grade and stimulation index are defined in text. See also Table I.

a detectable positive mismatch (donor positive–recipient negative); *C*, one positive mismatch; *D*, two or more positive mismatches. It is clear that excellent correlation existed between *HL-A* compatibility and nonstimulating cultures but thereafter there was no significant correlation for match-grades *B–D* and the MLC response.

When allele matching was analyzed, the expected distinction between *HL-A* compatibility and incompatibility was observed by both tests (Fig. 2 and Table I). Despite the tendency of mixed cultures between pairs matched for one allele to stimulate to a low degree ($F < 5$) there was great variability. No significant difference was discerned with respect to the two-allele disparate group.

TABLE I

Relationship of MLC Response to Match-Grade and Shared Allelism

Allele	*F*: stimulation index			
Match-grade:	*A*	*B*	*C*	*D*
(*P*)*	1.32			
	(<0.001)	16.80		
	(<0.005)	(>0.05)	19.97	
	(<0.001)	(>0.05)	(>0.05)	33.83
Shared alleles:	2	1	0	
(*P*)*	1.32			
	(<0.0025)	23.25		
	(<0.001)	(>0.05)	33.38	

* Probabilities are arranged to compare *F* values on corresponding vertical and horizontal axes.

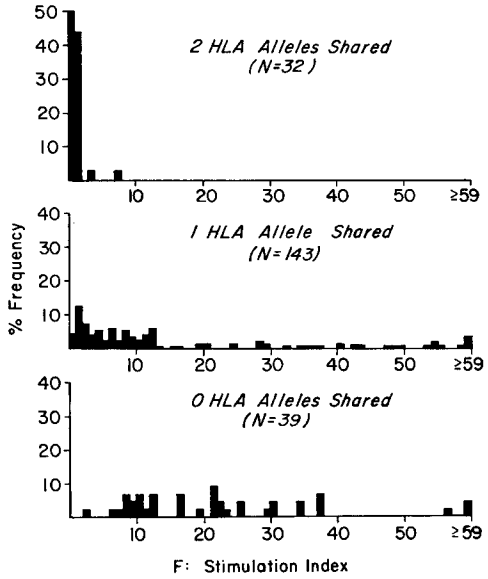

FIG. 2. Relationship of MLC to allele match-grade in 214 cultures performed within 16 families. See also Table I.

For the purposes of this investigation, another classification of histocompatibility was devised and termed the incompatibility number (IN). This classification gives weighted preference to allele matching and also considers mismatched and probably mismatched antigens. In this scale 0 indicates *HL-A*

compatibility for both alleles; 1 and 2 both reflect one shared allele, but in the former an antigen common to both cell types was identified in the unmatched allele and in the latter both antigens were mismatched; and 3, 4, and 5 encompass progressive degrees of antigen mismatching when neither allele was

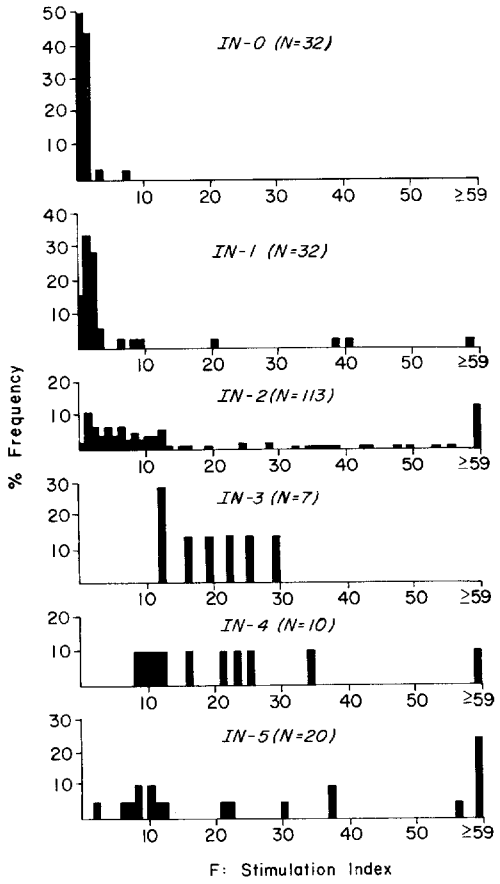

FIG. 3. Relationship of MLC to IN in 214 cultures performed within 16 families. IN is defined in text. See also Table II.

shared. Arbitrarily, 3 was assigned when only one antigen could be mismatched, 4 when two could be incompatible, and 5 when three or four antigens were not shared. The relationship between MLC and the incompatibility number proved to be very interesting because three groups were separable by these criteria (Fig. 3 and Table II). Once again, *HL-A* compatibility correlated with nonstimulating lymphocyte cultures, but those pairs sharing one allele plus another antigen also formed a definable group in which low and sometimes no

stimulation was common. Response to greater than $F = 10$ was comparatively uncommon, and group 1 not only proved to be statistically different from the pairs disparate for both alleles, i.e. 3, 4, and 5, but also different from those group 2 pairs sharing an allele that were incompatible for both antigens in the second allele.

Of related interest is the fact that group 2 ($F = 27.78$) did not significantly differ from groups 3 ($F = 27.15$), 4 ($F = 36.40$), and 5 ($F = 33.40$). The typing data were explored for the possibility that cross-reactive antigens might account for some of the low stimulation within a segment of the group. Surprisingly few examples were found and the occurrence was just as frequent between the pairs that stimulated to a higher degree. The single case of low

TABLE II

Relationship of MLC Response to Incompatibility Number

	F: stimulation index					
Incompatibility No.:	0	1	2	3	4	5
(*P*)*	1.32					
	(<0.02)	9.34				
	(<0.005)	(<0.02)	27.78			
	(<0.001)	(<0.05)	(>0.05)	22.15		
	(<0.001)	(<0.005)	(>0.05)	(>0.05)	36.40	
	(<0.001)	(<0.005)	(>0.05)	(>0.05)	(>0.05)	33.40

* Probabilities are arranged to compare *F* values on corresponding vertical and horizontal axes.

stimulation in group 5, $F = 2.36$, was found to be similar on retesting and could not be explained by known cross-reactive antigens.

Within a given family certain alleles seemed to exert more influence on the degree of stimulation than others. Family I-45 (Figs. 4 and 5 and Table III) was particularly informative because of the availability of three pairs of *HL-A*-compatible siblings in one generation plus three third-generation sons and the fact that the range of stimulation was so clearly marked. The genotypic expression of haplotypes is illustrated in Fig. 4. This family was also typed for *HL-A* antigens by Dr. Terasaki who provided identification of the haplotype *W-19* + *W-10* but otherwise was in accord with our typing. Within the second generation those siblings sharing the allele *HL-A3* + *W-15* (Fig. 4) and differing only by *HL-A1* vs. *HL-A2* in the second allele, i.e. IN-1, were all mutually nonstimulating in culture (Fig. 5). Evidence of their capacity to respond, however, was demonstrated by the reaction of siblings B_2 and B_3 who differed by both alleles and to C_1, a son of P who differed by his unrelated maternal allele but shared the *HL-A3* + *W-15* haplotype.

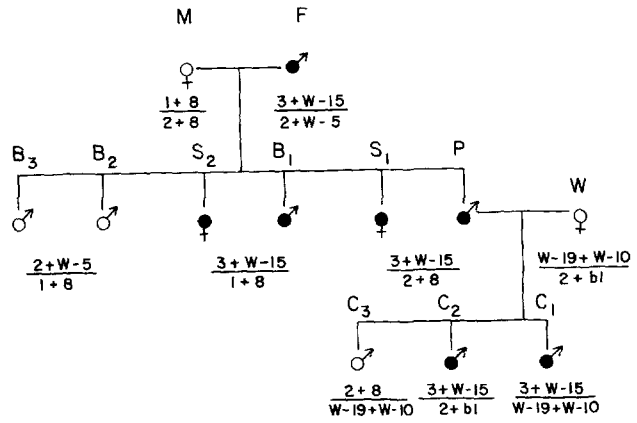

FIG. 4. Genetic inheritance of *HL-A* antigens in three generations of family I-45. Members sharing haplotype *HL-A 3 + W-15* are indicated by solid symbols.

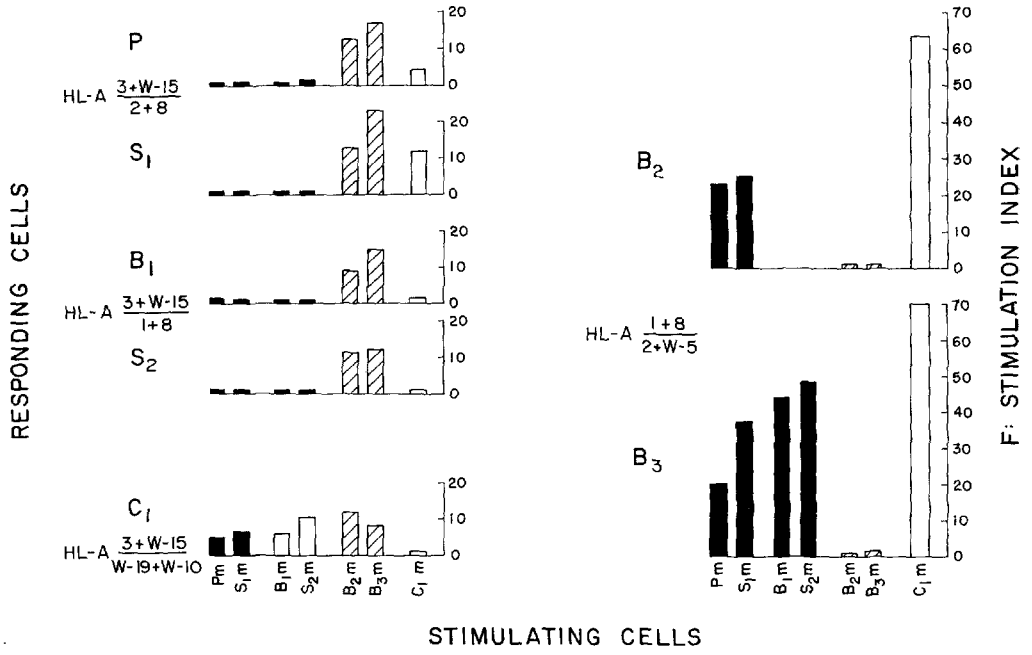

FIG. 5. Relationship of MLC to *HL-A* haplotypes in family I-45. Note non stimulation between siblings P, S_1 and B_1, S_2 despite incompatibility for *HL-A 1* and *2* between P, S_1, and B_1, S_2.

Since it was possible that the low stimulation was related to an inadequate number of mitomycin-treated stimulating cells, the tests were repeated using three times as many stimulating cells. One of each pair of siblings from the primary generation was retested as well as the sons and wife of P (Table III).

Despite the increase in stimulating cells, mutual nonstimulation between P and B_1 was again demonstrated, but nonstimulation was also found in the second generation, C_1 and C_2, who shared haplotype *HL-A3 + W-15* but differed with respect to the second allele. This is in sharp contrast to the strong response these same cells had with the other more genetically divergent cells.

Family I-75 afforded the opportunity to investigate the MLC reactivity between sets of all four possible *HL-A* alleles (Fig. 6 and Table IV). Typing

TABLE III

Stimulation Indexes in Augmented MLC in Family I-45

Responding cells (R)*		Stimulating cells (Sm)						
HL-A haplotypes	Cell donor	Pm	B_1m	B_2m	C_1m	C_2m	C_3m	W
3 + W-15 / 2 + 8	P	1.0	1.4	21.9	13.3	9.0	10.9	11.1
3 + W-15 / 1 + 8	B_1	1.2	1.0	86.9	93.2	32.8	114.5	173.5
1 + 8 / 2 + W-5	B_2	80.3	265.6	1.0	202.0	78.0	231.0	237.0
3 + W-15 / W-19 + W-10	C_1	18.2	22.2	51.8	1.0	0.7	18.4	27.4
3 + W-15 / 2 + b1‡	C_2	14.7	23.4	—	0.3	1.0	7.4	15.4
2 + 8 / W-19 + W-10	C_3	27.3	37.7	74.9	49.1	11.8	1.0	41.4
W-19 + W-10 / 2 + b1‡	W	184.4	96.0	169.0	68.0	10.0	81.0	1.0
		Stimulation index						

* R = P, patient; B_1 and B_2, siblings; C_1, C_2, C_3, sons; W, wife.

‡ *b1* = antigen not demonstrated.

indicated that the maternal haplotypes were *HL-A 2 + 12(A), 1 + 8(B)* and the paternal were *HL-A 10 + 8(C)* and *10 + W-17(D)*. The alleles and respective haplotypes are charted according to their incompatibility numbers in Table IV. In addition, the potentially stimulating *HL-A* antigens are indicated for each set of cultures. Fig. 6 demonstrates the *F* values where each vertical segment illustrates the reactions of a pair of *HL-A*–compatible cells and each point represents the mean response of these cells to mitomycin-treated cells obtained from both members of the incompatible pairs. The data are presented in this fashion in order to demonstrate the relative response of each pair of responding cells in the face of considerable variability in their absolute stimula-

tion by allogeneic cells. In all cases cultures between the *HL-A*-compatible siblings were not stimulated and are not depicted.

Although the IN-1 cultures were stimulated in every instance, the responses were always lower than either the IN-2 or IN-4 reactions with the same responding cells. With the exceptions of the *AC-BDm* and all *AD* reactions, the difference was statistically significant ($P < 0.05$). Of considerable importance is the fact that *BD-BCm* stimulated to the same degree as the other IN-1 cultures, yet there was no demonstrable *HL-A* antigen incompatibility that could account for the result.

TABLE IV

Relationship of HL-A Inheritance to Relative MLC Response in Family I-75

Incompatibility number*	*HL-A* genotypes		Mixed cultures		
	Alleles	Haplotypes	Responding and stimulating cells	*HL-A* antigen mismatch	Response‡
1	*AC*	*2 + 12, 10 + 8*	*AC-ADm*	*W-17*	L
	AD	*2 + 12, 10 + W-17*	*AD-ACm*	*8*	L
	BC	*1 + 8, 10 + 8*	*BC-BDm*	*W-17*	L
	BD	*1 + 8, 10 + W-17*	*BD-BCm*	None	L
2	*AC*	*2 + 12, 10 + 8*	*AC-BCm*	*1*	G
	BC	*1 + 8, 10 + 8*	*BC-ACm*	*2, 12*	G
	AD	*2 + 12, 10 + W-17*	*AD-BDm*	*1, 8*	I
	BD	*1 + 8, 10 + W-17*	*BD-ADm*	*2, 12*	I
4	*AC*	*2 + 12, 10 + 8*	*AC-BDm*	*1, W-17*	I
	BD	*1 + 8, 10 + W-17*	*BD-ACm*	*2, 12*	I
	AD	*2 + 12, 10 + W-17*	*AD-BCm*	*1, 8*	G
	BC	*1 + 8, 10 + 8*	*BC-ADm*	*2, 12, W-17*	G

* Incompatibility number defined in text.

‡ Relative response: L = least, I = intermediate, G = greatest, in relationship to all cultures performed with responding cell type.

The response of the IN-2 and IN-4 cultures within this family, however, reflect the same variability that was demonstrated in the composite study of 214 cultures in 16 other families. For example, intermediate stimulation (I) occurred within a pair that were incompatible for both alleles (*AC-BDm*) yet the greater response (G) was observed in the *AC-BCm* cultures where one allele was shared in common. This is even more striking in view of the demonstration that *HL-A1* was the only positively mismatched antigen in the latter cultures, whereas two *HL-A* antigens were mismatched in the former cultures.

Since these data seemed to indicate that the allele itself was more influential than *HL-A* antigens with respect to MLC reactions, it was helpful to be able to examine the question as to whether the origin of the same haplotype from

TABLE V

Effect of Common Parental Haplotype on MLC Response

Family	Responding cell (R)*		Stimulating cells (Sm)				
	Cell donor	*HL-A* haplotypes	Mm	Fm	Pm	B_2m	B_1m
I-50	M	*1 + 8, 11 + W-17*	1.0	1.7	6.6	1.9	5.2
	F	*1 + 8, 11 + b1*	9.0	1.0	1.5	1.1	1.5
	P	*1 + 8, 11 + b1*	8.2	1.8	1.0	1.7	4.3
	B_2	*1 + 8, 11 + b1*	3.3	0.5	1.2	1.0	1.3
	B_1	*11 + W-17, 11 + b1*	10.5	2.7	2.1	2.9	1.0
			Stimulation index				

* R = P, patient; B_1 and B_2, siblings; M, mother; F, father.

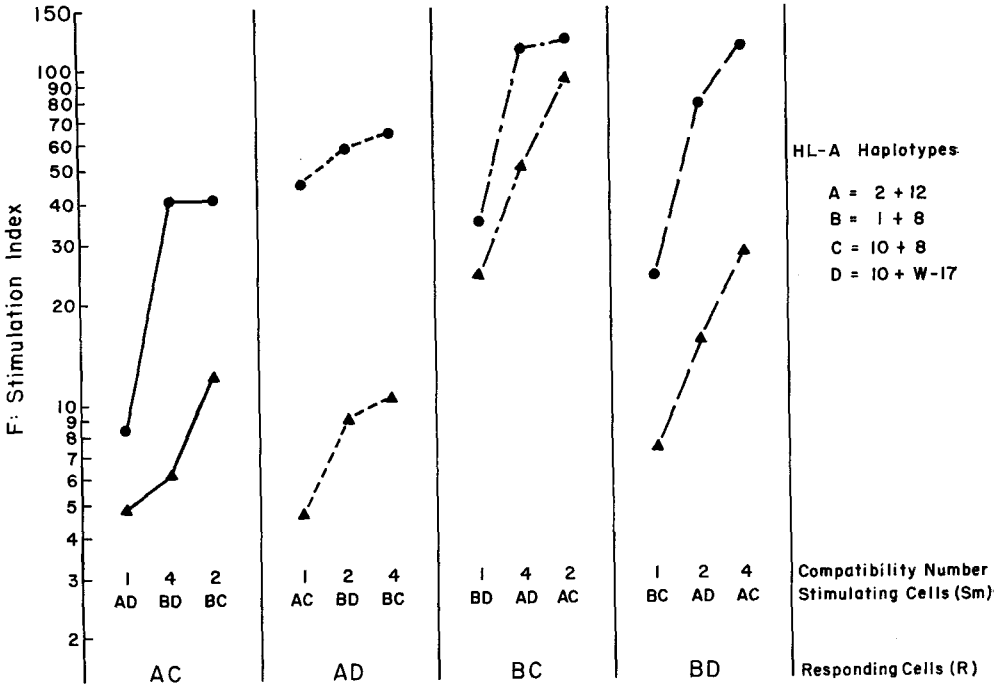

FIG. 6. Relationship of MLC to *HL-A* haplotypes in family I-75. Each division represents the response of a pair of *HL-A*-compatible siblings to a pair of siblings differing by all possible haplotypes within this family. The incompatibility number is defined in the text.

unrelated individuals would affect the response. Although there was no evidence of consanguinity, the mother and father of family I-50 both possessed the common haplotype *HL-A 1 + 8* (Table V). Two siblings inherited the maternal *1 + 8* haplotype and thus appeared to be *HL-A* compatible with the

father. MLC comparing these three cell donors were all nonstimulating and a subsequent renal transplant from the father to P has maintained excellent function with only one very mild rejection episode through 16 months of observation.

DISCUSSION

Before it was possible to ascertain the significance of these results, it was necessary to examine a number of nonspecific factors that influence lymphocyte cultures. It is well known that variability occurs in mixed cultures for apparently nongenetic reasons relating to the capacity of the responding cell to react to antigenic stimuli and the stimulating cell to evoke a response (2, 7). These phenomena were clearly seen when reactions were compared between *HL-A*-compatible siblings on more than one occasion such as in families I-45 and I-75, but we have observed that the qualitative relationships persist on repeated analysis despite quantitative change.

Another factor that may obscure the MLC reaction occurs when culture conditions are inadequate to demonstrate stimulation. In this regard it was important to establish some means of determining an expected level of response to a nonspecific stimulus and to a population of allogeneic cells. Originally, we relied upon the use of unrelated control stimulating and responding cells but it soon became apparent that a consistent reaction could not always be predicted. Bach et al. (2, 15) have demonstrated this variability by showing a spectrum of response to unrelated cells. Therefore, we have relied in this study on the PHA response (9) and the general effect and reaction of a cell type in multiple cultures between four and usually more family members to help distinguish artifactual data.

Since a critical aspect of this report deals with the group of very low and even nonstimulating cultures from *HL-A*-incompatible pairs, it is important to compare the sensitivity of our methods with other published results. To simplify the expression of the data we chose to use a stimulation index (F) comparing the reaction of a mixed culture with the response of the responding cell in culture with its own mitomycin-treated type. These control cultures varied about a range of 100–2000 cpm revealing that the reported F values reflected mixed culture responses of over 100,000 cpm in many instances. This compares very favorably with any of the values presented in the cited references. Although Bach (15) has recently reemphasized that apparent nonstimulation can be a function of culture conditions especially with respect to the number of mitomycin-treated stimulating cells, we have not found that increasing the dosage threefold has so altered the response as to convert apparent nonstimulation into stimulation. Family I-45 represents such an example. Low stimulation between *HL-A*-incompatible cells, moreover, has been previously reported by Seigler et al. (7).

Within these limitations, we confirm that there is a relationship between

inheritance of *HL-A* antigens and genetic factors determining MLC reactions. This inheritance, however, would appear to be more complex than a simple expression of *HL-A* antigen incompatibility or correspondence with the number of shared alleles of this locus. In both cases it is clear that great variability existed within groups defined by either criteria. The data, however, distinguish a group that share a common haplotype plus one antigen of the second unmatched haplotype that either stimulate to a relatively low level or not at all in mixed cultures. It was also apparent that MLC did not distinguish between those pairs sharing one common and one completely incompatible haplotype from the three groups, IN-3, 4, 5, in which neither haplotype was identical. It is of considerable interest that Dausset and Hors (5) have reported 92% survival for haplo-identical parent-to-child or sibling-to-sibling renal grafts whose calculated degree of compatibility was roughly comparable to our IN-1 group, whereas survival was reduced to 50% in the group of haplo-identical related pairs with a greater degree of incompatibility.

Alternate hypotheses based on the existence of additional unidentified segregating series, discordance of the loci coding for the mixed lymphocyte response and *HL-A* antigens, or an undetected role of non-*HL-A* antigenic stimulation may be advanced to explain our data. The results, however, favor the concept that the haplotype is the principle immunogen in MLC reactions, and partial compatibility when one antigen is shared reduces the immunogenicity of this unit. If the haplotype functions structurally as the immunogen, complete incompatibility for two haplotypes might not provoke any greater response than one, providing there was no similarity between any of the haplotypes involved. Certainly, there is no clear evidence from other complex systems such as the Rh human erythrocyte antigens that incompatibility for two antigens induces any greater antibody response or more severe reaction than one. From this it would be predicted that cells derived from unrelated individuals typing for the same four antigens could stimulate in culture if the haplotypes were not identical. Examples of stimulation have, in fact, exceeded examples of nonstimulation in studies from three different groups (8, 10, 16). Unfortunately, genotyping for haplotype identity was not included in any of these reports. Our studies have revealed one example of stimulation in which the mitomycin-treated cells differed by one haplotype but no positively mismatched antigens. A further source of potential stimulation arises from the possibility that a haplotype, i.e. *HL-A 1 + 8*, from one person may be immunogenetically dissimilar to that of another. For this reason, we were fortunate to encounter an example, I-50, where the same haplotype from unrelated individuals behaved compatibly in culture and thus far in the course of transplantation. It will be important to investigate this point much further with respect to other examples of this and other haplotype compatibilities when compared with unrelated cell types.

Recently, it has been suggested that cadaveric renal transplants should be

matched on the basis of identity for at least one *HL-A* haplotype. If MLC can be taken as supportive evidence of graft compatibility, the present study would suggest that an effort be made to select those transplants where one haplotype plus one antigen of the unmatched haplotype is shared. This necessitates genotyping the cadaveric donor as well as all recipients. The number of potentially acceptable kidneys that fit these criteria would be considerably greater than if four matched antigens were required. Furthermore, compatibility of all four antigens does not guarantee haplotype identity and our data suggest that this is the more important determinant in mixed cultures. Consequently, we have undertaken to genotype all transplant donors and recipients and selection will be made on the basis of IN-0 or 1 whenever possible.

SUMMARY

The influence of varying degrees of incompatibility for *HL-A* antigens on one-way mixed lymphocyte cultures (MLC) has been investigated. Reactions have been compared to a simple expression of *HL-A* antigens, allele compatibility, and a proposal considering the potential influence of antigen matching in relationship to allele compatibility.

As expected, *HL-A* compatibility was associated with nonstimulated cultures, but significant correlation was not observed when incompatibility was expressed in terms of *HL-A* antigen or allele mismatching.

When the relationship of both was considered, however, a second distinctive group was demonstrated that shared one allele plus one antigen of the second allele. Within this group no stimulation, even with augmented culture conditions, was observed in some families. Employing these same criteria, there was no significant difference in the MLC response in those groups that were incompatible for both alleles regardless of the number of matched antigens or the group that shared an allele but differed by both antigens of the second allele.

These results support the concept of an intimate relationship between the loci coding for *HL-A* antigens and mixed culture reactions. They suggest that *HL-A* haplotype incompatibility acting as a unit is the primary stimulus of the MLC response, and that the immunogenicity of the haplotype also relates to whether or not one antigen is common to the stimulating and responding cell.

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