QUANTITATIVE ASSAY OF ANTIGENIC DISPARITY AT *HL-A*— THE MAJOR HISTOCOMPATIBILITY LOCUS IN MAN*

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The polymorphism at *HL-A* is so extensive (1–3) that the likelihood of finding two individuals, other than siblings, who are genotypically identical is very small. For transplantation programs, it may therefore become necessary to measure degrees of antigenic disparity at *HL-A* to predict the probable extent of recipient reaction against the foreign transplantation antigens present on donor tissue.

At present there are two approaches to histocompatibility testing. Typing procedures make use of suitable isoimmune antisera to define specific antigens responsible for incompatibility, but cannot determine, except as inferred from the number of antigens by which two individuals differ, the "degree" of antigenic disparity between them. Matching tests such as the mixed leukocyte culture (MLC) test cannot enumerate specific antigenic differences, but do allow assay of antigenic disparity based on the responsiveness of lymphocytes of the recipient to foreign antigens, thus probably providing a physiological measure of incompatibility.

Previous studies using one-way stimulation in MLC tests have allowed definition of nonstimulation, or MLC identity (4), in some mixed pairs of cells, and have permitted a genetic analysis based on the percentage of MLC-identical individuals of different genetic relationship (5). However, for this test to qualify as a matching procedure able to meaningfully detect different degrees of incompatibility, different amounts of stimulation in MLC tests must relate to different degrees of antigenic disparity. If stimulation represents incompatibility at *HL-A*, it would be difficult to correlate different degrees of stimulation in MLC tests with skin graft survival, since the majority of grafts between individuals differing at the major locus are rejected in 8–13 days. With the difficulties in accurately evaluating the exact time of graft rejection and the varying contribution of additive minor loci differences (6, 7) to the exact time of rejection, a more sensitive criterion to judge antigenic disparity at *HL-A* alone seems desirable.

A genotype can be proposed for siblings in a family by analysis of leukocyte antigens. At a very polymorphic locus such as *HL-A*, both parents will usually be hetero-

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zygous for different alleles and four groups of siblings can be identified. If the father's alleles are designated a and b, and the mother's c and d, then the four sibling groups will be characterized by allelic combinations ac, ad, bc, and bd. Siblings within each group do not stimulate in MLC tests and possess identical leukocyte antigens (with very few exceptions). With any group as reference (e.g. ac), two groups (ad and bc) differ by one allele while the remaining group (bd) has no common allelic inheritance, and thus differs by both alleles. Within such a sibship, a sibling differing by both alleles from the prospective recipient would necessarily show the maximum antigenic disparity within that sibship (assuming no null allele). Depending on which sibling is chosen as responder, all other siblings in the family differ by either "no allele," "one allele," or "two alleles."

If the MLC test is capable of detecting different degrees of incompatibility, a simple prediction can be made when cells of different sibling pairs are mixed in culture. With a given sibling as responder, cells of siblings differing by two alleles from the responder should give greater stimulation than cells of siblings differing by only one allele from the responder. This paper describes the results of studies in five families, each tested from two to four times, in which such predictions were realized. A total of 15 experiments are included in this series.

Method

Mixed Lymphocyte Culture Technique.—The one-way MLC technique has been previously described in detail (4). In these experiments, several concentrations of stimulating cells are tested with a constant concentration of responding cells. The responding cell concentration is held constant at 0.3×10^6 mononuclear cells per milliliter while stimulating cells are usually present at a final concentration of 0.25, 0.5, 0.75, and 1.0×10^6 leukocytes per milliliter. Occasionally other concentrations of stimulating cells are used. Replicate (duplicate or triplicate) cultures of 2.5 ml are made at each concentration for each pair tested. In some experiments, cells to be used as stimulating cells are first purified after the method of Rabinowitz (8) to obtain a lymphocyte-rich suspension. In most experiments however, this purification step is omitted.

Leukocyte Typing.—The method of leukocyte typing using cytotoxic antisera, and the procedure for assignment of genotypes within a family are described in the preceding paper (9).

RESULTS

Figs. 1, 2, and 3 show the results of MLC testing in the H family (Table I in the preceding paper). The letters A, B, C, and so forth refer to siblings; X and Y to parents; and Z to an unrelated individual. It is assumed, because of the polymorphic nature of *HL-A*, that parents will, in the vast majority of cases, differ from their children by one allele and that unrelated individuals will differ by two alleles. The subscript m refers to cells treated with mitomycin C.

In Fig. 1, A is the responding sibling; C is a sibling who shares the same *HL-A* alleles as A; E is a sibling who differs by one allele (shares the c allele but differs by the a allele); X is a parent (thereby differing by one allele); G is

a sibling differing by both alleles; and Z is unrelated to A. The maximum stimulation seen in this experiment, as judged by comparing the different cell mixtures, is obtained in the mixture AG_m.

Fig. 2 shows the results obtained in a second experiment with this family. The protocol is exactly the same as that in the first experiment except that the responding individual is sibling G. A and C are, therefore, siblings differing by two alleles with respect to G, and cell mixtures GA_m and GC_m give the

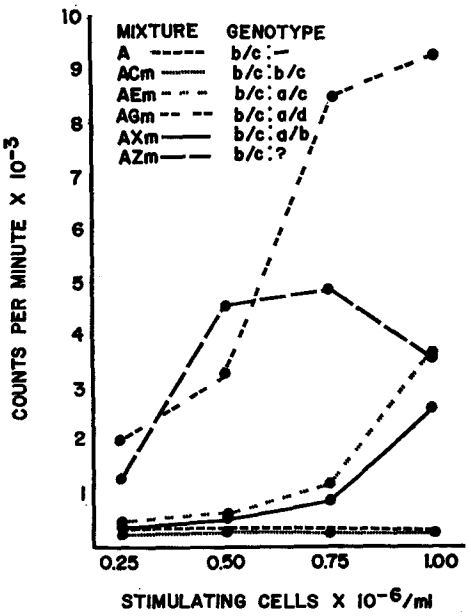

FIG. 1. Result of an MLC test in the H family. Sibling A is the responder. Cell mixture AG_m shows maximum stimulation whereas mixture AC_m is nonstimulatory. A's cells cultured alone serve as the control.

maximum stimulation seen in this experiment. Cells of C, which gave no stimulation when cultured with cells of A in the experiment shown in Fig. 1, in this experiment give maximum stimulation. Siblings E and F are siblings who differ by one allele with respect to G (as they do with respect to A; however with respect to G they differ by the c allele and share the a), and mixtures of cells of these siblings with cells of G give intermediate stimulation. Siblings G and I, who are assigned identical parental alleles based on their antigenic profiles, are clearly different as discussed in the previous paper (9). This difference is again demonstrated in Fig. 2.

Fig. 3 shows the results of a third experiment. Sibling C is now the responder; D is a sibling sharing both alleles with C; and X and Y are parents.

In this case, since siblings G and I, who each seem to differ from C by two alleles, are known to differ from each other, we could only predict that one of these siblings would show the maximum stimulation with cells of C. As shown in Fig. 3, the mixture CG_m did give the maximum stimulation in this experiment whereas CI_m only provided intermediate stimulation.

Four additional families were studied in the same manner as the H family. In two of these additional families unequivocal genotypes could be proposed as

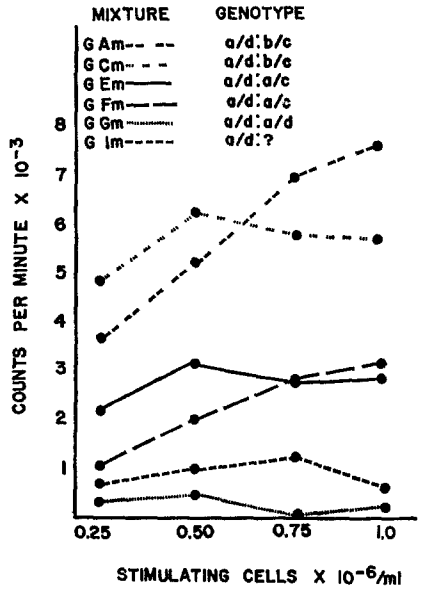

FIG. 2. Results of a second MLC test in the H family. Sibling G is the responding individual and cell mixtures GA_m and GC_m show maximum stimulation. G stimulating cells (G_m) tested against G-responding cells serve as the control.

for the H family. In the remaining two the leukocyte antigen data are somewhat ambiguous in dictating a unique genotype. Table I shows the results of leukocyte typing and the proposed genotypes of the Q family. Although two possible genotypes are given, the weight of evidence suggests that the first genotype proposed is the correct one.

The MLC data in this family are quite unambiguous. Two of the four MLC tests done are shown in Figs. 4 and 5. In the first experiment (Fig. 4) sibling C is the responder. All cell mixtures from the sibling pair CA_m clearly give the maximum response seen in this experiment. This result is consistent with either of the proposed genotypes. In the first instance, siblings C and A differ from each other by two alleles, whereas in the second neither sibling B nor sibling A differs from C by both alleles and no prediction regarding maximum

stimulation can be made. The shape of the dose-response curve (CA_m) is different from those curves presented thus far. Such a dose-response relationship has been noted on several occasions in our family studies. Fig. 5 gives the results of MLC tests when sibling A is used as the responding individual. Cells of sibling C now give maximum stimulation, as would be expected if the first proposed genotype is indeed the correct one, since siblings A and C differ from

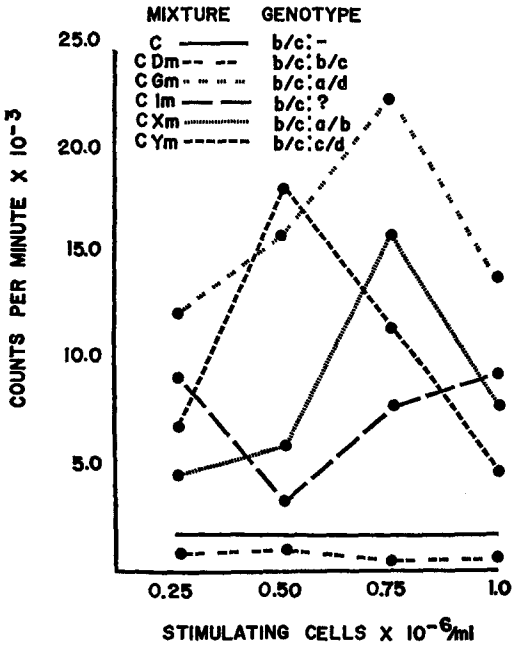

FIG. 3. A third MLC test in the H family. Identifying letters refer to the same individuals as in Figs. 1 and 2. Sibling C is the responder with cell mixture CG_m showing maximum stimulation. C cells cultured alone serve as the control.

each other by both alleles in this scheme. If the second scheme were correct, sibling B would differ from A by two alleles. The form of the dose-response curve AC_m is characterized by initial high stimulation followed by decreasing stimulation provided by increasing numbers of stimulating cells. Such a response pattern has only been noted in mixtures of cells of either unrelated individuals or siblings who, as determined by genotyping, differ at *HL-A* by two alleles.

In this series of experiments, several concentrations of stimulating cells are used in any given test. For each combination of stimulating and responding cells, four values of "stimulation" (for the four doses of stimulating cells used) must be considered in any quantitative expression reflecting the degree of response in that combination. High stimulation with only small numbers of

TABLE I

Leukocyte typing and proposed genotyping for the Q family. Antisera listed in parentheses by each genotype proposal are those antisera which are not accounted for by that particular proposal.

Antisera	Father	Mother	Siblings		
			A	B	C
RIL	+++	0	0	+++	+++
DAL	+++	0	0	+	++
RA	++	0	0	+++	++
RB	++	0	++	0	0
PIG	0	++	0	0	++
KH	0	++	0	0	++
NW	±	++	0	0	++
THA	+++	+++	+++	0	++
DK	+	+++	+	0	+++
COU	0	++++	+++	0	+++
ENN	0	+++	+++	0 −±	±

Father			Mother			Sibling		Genotype
a	b		c	d			A	b/d
			+PIG			[COU]	B	a/d
+RIL			+DK			[ENN]	C	a/c
+DAL		+DK	+KH					
+RA		+THA	+NW					
		+RB	+THA					

Father			Mother			Alternative genotype		
						Sibling		Genotype
a	b		c	d		[PIG]	A	b/c
+RIL						[KH]	B	a/d
+DAL		+DK	+COU			[NW]	C	a/c
+RA		+THA	+ENN					
		+RB	+THA					
			+DK					

stimulating cells appears to reflect greater antigenic disparity than an equal amount of stimulation requiring four times as many stimulating cells. Therefore, in the expression of the combined values for any one combination (for example, AB_m), the stimulation seen with 0.25×10^6 stimulating cells per milliliter is weighted more heavily than that seen with a higher concentration of stimulating cells. An arbitrary weighting method is simply to multiply the counts per minute incorporated at any concentration of stimulating cells by the reciprocal of the

number of stimulating cells expressed in millions. Thus, the counts per minute obtained at 0.25×10^6 stimulating cells per milliliter is multiplied by four, that obtained at 0.5×10^6 stimulating cells is multiplied by two, and so on; and the four products are added together to give a final quantitative expression of stimulation. This method of quantitation is completely arbitrary, and its utility remains to be determined on the basis of further experiments both in families and among unrelated individuals.

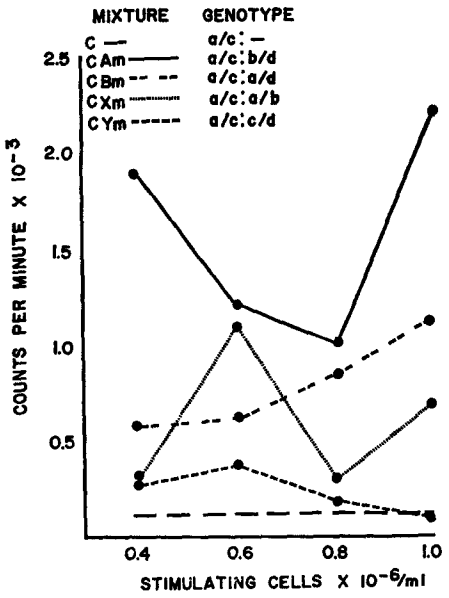

FIG. 4. Results of an MLC test in the G family. Sibling C is the responder with cell mixture CA_m showing the greatest stimulation. Stimulating cell concentrations of 0.4, 0.6, 0.8, and 1.0×10^6 leukocytes per milliliter were used in this experiment. C cells cultured alone serve as the control.

Table II gives the results of such calculations for each experiment in the present series. In every experiment, with one exception, cells of the sibling differing from the responder by two alleles give maximum stimulation to cells of that responder. In four of the families, different responding siblings are used in succeeding experiments. Such changes result in siblings who had formerly differed from the responder by one allele now being the ones who differ from the new responder by two alleles. In all such cases, cells of those siblings now differing from the new responder by two alleles give maximum stimulation to cells of that responder. However, if siblings who differ from a responder by only a single allele (such as AC_m and AD_m in the Z family) are compared, then in some experiments the rank order of such siblings changes.

For example, in experiment 14, cells of sibling C stimulate the cells of sibling A more than do the cells of sibling D. In experiment 15, the order is reversed. On the other hand, in several other experiments even the rank orders of siblings differing by only one allele are maintained.

Fig. 6 gives the results of that experiment in the J family which gave the

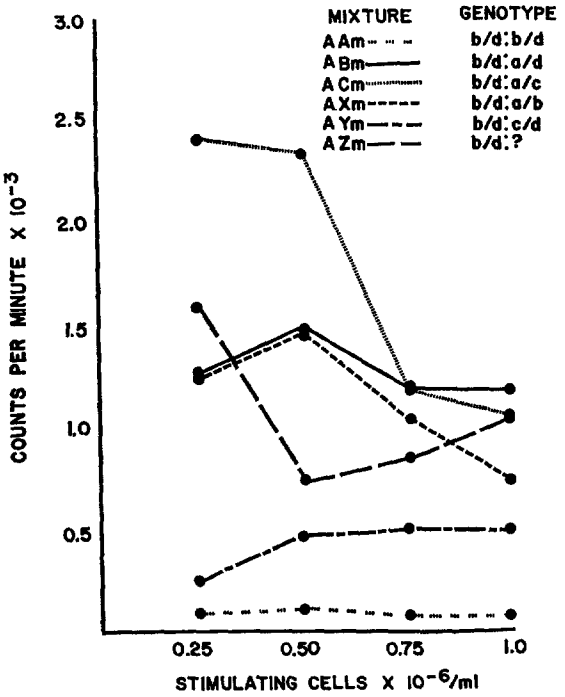

FIG. 5. MLC results of a second experiment in the Q family. Sibling A is the responder with cell mixture AC_m showing maximum stimulation. The dose response relationship of cell mixture AC_m is characterized by initial high stimulation followed by decreasing stimulation provided by increasing numbers of stimulating cells. Purified stimulatory cells are used in this experiment. This same dose response relationship has been observed in experiments where nonpurified stimulating cells are used. Cell mixture AA_m serves as the control.

single exception. In this experiment, sibling D is the responding individual, with siblings B and C differing from the responder by two alleles. As expected, these cell mixtures (DB_m and DC_m) give the maximum stimulation observed in the sibship. However, cell mixture DY_m, which involves stimulation of D cells by those of the mother, gives stimulation which is at least as great as that observed in the mixtures of cells differing by two alleles. (The mother must necessarily differ from the responding siblings by only one allele.) In the two additional experiments done in this family, the results are completely con-

TABLE II

Results of the 15 independent MLC tests comprising the present series. Cell mixtures with their respective genotypes are listed as is the numerical value obtained for each cell mixture when the data are transformed as indicated in the text. For the Q and Z families, in whom the leukocyte antigen data are somewhat ambiguous in dictating unique genotypes, only those genotypes most favored by this leukocyte antigen data are listed.

H family			G family			J family			Q family			Z family		
Cell mixture	Genotype	Value	Cell mixture	Genotype	Value	Cell mixture	Genotype	Value	Cell mixture	Genotype	Value	Cell mixture	Genotype	Value
Exp. 1			*Exp. 4*			*Exp. 7*			*Exp. 10*			*Exp. 14*		
AC_m	b/c:b/c	2401	AX_m	b/d:a/b	21139	EF_m	b/c:b/c	703	CX_m	a/c:a/b	1684	AY_m	a/c:c/d	6438
AE_m	b/c:a/c	8594	AY_m	b/d:c/d	21816	EC_m	b/c:b/d	4552	CY_m	a/c:c/d	3802	AB_m	a/c:b/d	28008
AX_m	b/c:a/b	6164	AB_m	b/d:a/d	28006	EB_m	b/c:b/d	7534	CB_m	a/c:a/d	4674	AC_m	a/c:b/c	7066
AG_m	b/c:a/d	35290	AC_m	b/d:a/d	29729	ED_m	b/c:a/c	4804	CA_m	a/c:b/d	11293	AD_m	a/c:a/d	2847
AZ_m	b/c:?	24009	AD_m	b/d:a/c	33163	EA_m	b/c:a/d	11035	*Exp. 11*			*Exp. 15*		
Exp. 2			AE_m	b/d:b/d	3751	EX_m	b/c:a/b	7858	CX_m	a/c:a/b	3453	AY_m	a/c:c/d	8863
GI_m	a/d:a/d	7266	*Exp. 5*			EY_m	b/c:c/d	8352	CY_m	a/c:c/d	5383	AB_m	a/c:b/d	23932
GF_m	a/d:a/c	15952	BA_m	a/d:b/d	2541	*Exp. 8*			CB_m	a/c:a/d	6826	AC_m	a/c:b/c	6200
GE_m	a/d:a/c	30163	BX_m	a/d:a/b	4443	FE_m	b/c:b/c	595	CA_m	a/c:b/d	10343	AD_m	a/c:a/d	7513
GC_m	a/d:b/c	47059	BY_m	a/d:c/d	15195	FD_m	b/c:a/c	2852	*Exp. 12*					
GA_m	a/d:b/c	41595	BC_m	a/d:a/d	483	FC_m	b/c:b/d	1467	CX_m	a/c:a/b	11690			
Exp. 3			*Exp. 6*			FB_m	b/c:b/d	1990	CY_m	a/c:c/d	15921			
CX_m	b/c:a/b	58922	AX_m	b/d:a/b	12242	FA_m	b/c:a/d	4992	CB_m	a/c:a/d	16669			
CY_m	b/c:c/d	84165	AY_m	b/d:c/d	27180	FX_m	b/c:a/b	2382	CA_m	a/c:b/d	17222			
CD_m	b/c:b/c	7236	AB_m	b/d:a/d	18022	FY_m	b/c:c/d	2193	*Exp 13*					
CI_m	b/c:a/d	63470	AC_m	b/d:a/d	58845	*Exp. 9*			AC_m	b/d:a/c	17934			
CG_m	b/c:a/d	123934	AE_m	b/d:b/d	1581	DF_m	a/c:b/c	4009	AX_m	b/d:a/b	10022			
						DE_m	a/c:b/c	3335	AY_m	b/d:c/d	3130			
						DC_m	a/c:b/d	5940	AB_m	b/d:a/d	10754			
						DB_m	a/c:b/d	7193						
						DA_m	a/c:a/d	2530						
						DX_m	a/c:a/b	5816						
						DY_m	a/c:c/d	7767						

sistent with expectations; the maximum stimulation is shown by sibling cell mixtures which differ by two alleles (Table II).

DISCUSSION

In MLC tests, lymphocytes of a potential recipient respond to foreign histocompatibility antigens present on cells of a potential donor by enlarging

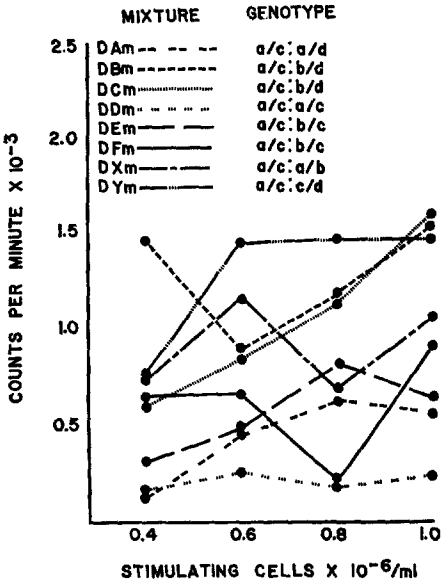

FIG. 6. Results of one MLC test in the J family showing the single exception noted in this series. Sibling D is the responder. Cell mixture DY_m shows maximum stimulation whereas cell mixture DB_m and DC_m are from siblings who differ by two alleles. Stimulating cell concentrations of 0.4, 0.6, 0.8, and 1.0 $\times$ 10^6 leukocytes per milliliter are used. D stimulating cells (D_m) tested against D-responding cells serve as the control.

and incorporating radioactive thymidine (stimulation). We have here presented evidence that different degrees of response in MLC tests are, by the criteria listed, meaningful in that they measure the amount of antigenic disparity between donor and recipient cells.

Results obtained with the MLC method used in this study have features which may account for earlier difficulties in attempts to quantify stimulation. In Fig. 1, cell mixtures AE_m and AX_m would be considered nonstimulatory if only stimulation values with concentrations of 0.25 and 0.5 $\times$ 10^6 cells per milliliter are considered. When several concentrations are used, including 1.0 $\times$ 10^6 stimulating cells per milliliter, these mixtures are in fact found to be stimulatory. These mixtures are different from the cell mixture AC_m in which

there is no stimulation at any of the concentrations of stimulating cells used (an MLC-identical mixture).

Mixture AC_m in Fig. 5 shows another pattern. In this case, maximum stimulation occurs at the lowest concentration of stimulating cells (0.25×10^6 cells per milliliter), and further increases in the number of stimulating cells result in "less" stimulation. Although it is difficult to interpret the meaning of such "inhibition," we have noted this pattern in the past and it does correlate with the most marked antigenic differences which one might expect at this locus.

The antigenic data on two of the families studied (Q and Z family) is ambiguous in suggesting a unique genotype. For each of these families, two alternative genotypes could be proposed, although in each case, the weight of evidence, based on antigenic phenotypes, suggests one in preference to the other. Mixed leukocyte culture test results were not ambiguous in either family as can be seen from Table II. In both cases they are consistent with that genotype proposal most favored by the leukocyte antigen data.

In one experiment there is a clear exception to our predictions. This is in the J family where cells of the mother stimulate more than do cells of siblings differing by two alleles. It can be seen from Table II, however, that this difference is very slight. Such an exception might be expected in the present state of the MLC method. In all of these families, while the general prediction has been realized, and stimulators who differ from the responder by two alleles can be differentiated from those who differ by one allele, it must be noted that these are rather gross differences. Smaller degrees of difference, such as between some siblings differing by only one allele, are probably not meaningful since such differences are not reproducible in all cases. Thus, if the mother's antigenic disparity from the responding child is fairly close to that of the siblings differing by two alleles from this responder, such an exception might be expected.

Even considering only those three families in which an unequivocal genotype could be proposed, it would be unreasonable to assume a correlation such as we have observed by chance alone. As shown in Table II, seven experiments were performed in these three families in which a two allele difference between responder and stimulating sibling was established. In experiment 1, the probability of choosing cell mixture AG_m as that mixture showing maximum stimulation by chance alone is $\frac{1}{4}$. (Cell mixture AZ_m is not considered as Z is an unrelated individual about whose cells no prediction can be made.) In experiment 2, the probability of choosing both cell mixtures GA_m and GC_m as those mixtures showing maximum stimulation is $\frac{1}{10}$ ($\frac{2}{5} \times \frac{1}{4}$). In experiment 3, mentioned above, we had a $\frac{2}{5}$ chance of preselecting the maximally stimulating cell mixture since we could only predict that one of two mixtures would stimulate the most. Each experiment has a different probability de-

pending on the number of individuals tested with a range for these seven experiments between $\frac{1}{4}$ and $\frac{1}{10}$, except for the unusual situation in experiment 3 where the probability is $\frac{2}{5}$. If we use the most conservative probability figure, i.e. $\frac{1}{4}$, for those six experiments in which the genotype of all relevant cell mixtures is known (which allows the greatest possibility of choosing the maximally stimulating cell mixtures by chance alone) the probability of choosing the correct mixture or mixtures in five out of six cases is $\frac{18}{4096}$ ($\frac{1}{228}$). In addition, in experiment 3, the maximally stimulating cell mixture was one of two, as predicted, even though there were five cell mixtures in the experiment. Thus, the most conservative probability of obtaining this correlation by chance alone in these seven experiments is approximately $\frac{1}{570}$. This correlation of maximum antigenic disparity and maximum stimulation is therefore highly significant.

In some of the cell mixtures known to differ by two alleles at *HL-A*, there is the empirical observation of high stimulation with low numbers of stimulating cells and less stimulation with increasing numbers of stimulating cells. In other cell mixtures the dose-response relationship is best described by a biphasic curve. The reasons for these reponse patterns are not understood. However, we present this modified technique for MLC tests, on the basis of the empirical correlations made and suggest it as a method to quantitate antigenic disparity at *HL-A*. Quantitation is expressed on a comparative scale within a single experiment and no attempt is made to compare counts per minute incorporated in different experiments. It must be stressed that the correlation we have shown indicates only that what are probably fairly major relative differences in antigenic disparity in the majority of cases can be detected with good reproducibility, and in such instances the amount of stimulation observed is immunogenetically meaningful.

SUMMARY

We have extended the method of one-way stimulation in mixed leukocyte culture tests as previously described to quantitate different degrees of stimulation. To demonstrate that the amount of stimulation is immunogenetically meaningful, siblings and parents in families in whom genotyping on the basis of leukocyte antigen data was possible were tested. The prediction that cells of siblings differing from the responding sibling by both alleles at *HL-A*, stimulate more than do cells of siblings differing by only one allele, was realized in every case. One exception, with cells of a parent, is discussed. It is stressed that the differences measured here are probably fairly strong ones in the majority of cases, and that lesser differences cannot yet be detected reproducibly.

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BIBLIOGRAPHY

1. Van Rood, J. J., A. Van Leeuwen, A. Schippers, W. H. Vooys, E. Fredericks, H. Balner, and J. G. Eernisse. 1965. Leukocyte groups. The normal lymphocyte transfer test and homograft sensitivity. *In* Histocompatibility Testing 1965. Munksgaard, Copenhagen. 37.
2. Dausset, J., P. Ivanyi, and D. Ivanyi. 1965. Tissue alloantigens in humans. Identification of a complex system (Hu-1). *In* Histocompatibility Testing 1965. Munksgaard, Copenhagen. 51.
3. Bach, F. H., and D. B. Amos. 1967. Hu-1: Major histocompatibility locus in man. *Science.* **156**:1506.
4. Bach, F. H., and N. K. Voynow. 1966. One-way stimulation in mixed leukocyte cultures. *Science.* **153**:545.
5. Bach, F. H. 1966. Mixed leukocyte cultures—their assay and genetics. *In* In Vitro II. Waverly Press, Inc., Baltimore. 32.
6. McKhann, C. F. 1964. Additive effect of multiple weak incompatibilities in transplantation immunity. *Nature.* **201**:937.
7. Graff, R. J., W. K. Silvers, R. E. Billingham, W. H. Hildermann, and G. D. Snell. 1966. The cumulative effective of histocompatibility antigens. *Transplantation.* **4**:605.
8. Rabinowitz, Y. 1964. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. *Blood.* **23**:811.
9. Amos, D. B., and F. H. Bach. 1968. Phenotypic expressions of the major histocompatibility locus in man (*HL-A*): Leukocyte antigens and mixed leukocyte culture reactivity. *J. Exptl. Med.* **128**:632.