GENETIC AND CELLULAR ASPECTS OF XENOGENEIC MIXED LEUKOCYTE CULTURE REACTION*

By KIRSTEN FISCHER LINDAHL‡ AND FRITZ H. BACH

(From the Immunobiology Research Center, Departments of Medical Genetics and Surgery, The University of Wisconsin, Madison, Wisconsin 53706)

Xenogeneic lymphocytes can induce proliferative responses in the mixed lymphocyte culture (MLC)[1] which are as strong as those induced by allogeneic lymphocytes (1). It is impossible to determine directly which lymphocyte antigens stimulate the xenogeneic MLC response. The recently described "primed lymphocyte typing" (PLT) assay (2) has made it possible to determine indirectly the genetic control of xenogeneic MLC. Sensitization in a mixed lymphocyte culture positively selects for memory cells which give a rapid secondary response upon restimulation with cells from the initial stimulator (3, 4). Studies in human families (2, 5–7) showed that restimulation is caused by the same LD determinants of the major histocompatibility complex (MHC) that induce primary MLC responses.

In this paper we present studies of human lymphocytes which were sensitized to cells of a single mouse strain and restimulated with cells from a variety of strains. By choosing combinations of primary and secondary stimulator which shared only certain parts of their genetic material, we could assay the relative importance of non-H-2 and various H-2 antigens for activation of human lymphocytes. The results suggest that the same antigens are important in allogeneic and xenogeneic MLC reactions. The ability of human leukocyte subpopulations to respond in xenogeneic MLC further suggest that the cellular requirements of allogeneic and xenogeneic MLC responses are the same.

Materials and Methods

Mice. Mice used in this study were bred in our own colony or purchased from The Jackson Laboratory, Bar Harbor, Maine (Table I).

Spleen Cells. Spleens were removed aseptically into phosphate-buffered saline (PBS) (Dulbecco's, Ca^{2+} and Mg^{2+} free, Grand Island Biological Company, Grand Island, New York), and the cells were pressed through a stainless steel wire screen. The suspension was allowed to settle and big sediments removed. Erythrocytes and dead cells were removed by hypotonic shock (8); the cells were resuspended in PBS and viable cells counted in eosin.

* This work was supported by National Institutes of Health grants AI-11576, CA-16836, and AI-08439 and National Foundation-March of Dimes grant CRBS 246. This is paper no. 1976 from the Laboratory of Genetics and paper no. 78 from the Immunobiology Research Center.

‡ Present Address: Institute of Genetics, 5 Köln 41, Weyertal 121, Federal Republic of Germany.

[1] *Abbreviations used in this paper:* E-RFC, cells forming rosettes with sheep erythrocytes; MLC, mixed leukocyte culture; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PLT, primed lymphocyte typing; PWM, pokeweed mitogen.

TABLE I
Mouse Strains Used in This Paper and Their H-2 Types

Mouse strain	*H-2* haplotype	Origin of *H-2* regions					
		K	*I-A*	*I-B*	*I-C*	*S*	*D*
C57BL/10	*b*	*b*	*b*	*b*	*b*	*b*	*b*
B10.D2 DBA/2J	*d*	*d*	*d*	*d*	*d*	*d*	*d*
AKR/J B10.BR	*k*	*k*	*k*	*k*	*k*	*k*	*k*
B10.G DBA/1J	*q*	*q*	*q*	*q*	*q*	*q*	*q*
A.SW/Sn B10.S	*s*	*s*	*s*	*s*	*s*	*s*	*s*
B10.A	*a*	*k*	*k*	*k* |	*d*	*d*	*d*
B10.A(2R)	*h2*	*k*	*k*	*k*	*d*	*d* |	*b*
B10.A(4R)	*h4*	*k*	*k* |	*b*	*b*	*b*	*b*
B10.A(5R)	*i5*	*b*	*b*	*b* |	*d*	*d*	*d*
B10.AKM	*m*	*k*	*k*	*k*	*k*	*k* |	*q*
B10.S(7R)	*t2*	*s*	*s*	*s*	*s*	*s* |	*d*
B10.HTT	*t3*	*s*	*s*	*s* |	*k*	*k*	*d*
AQR	*y1*	*q* |	*k*	*k*	*d*	*d*	*d*
B10.T(6R)	*y2*	*q*	*q*	*q*	*q*	*q* |	*d*

Vertical lines indicate position of cross-over.

Human Lymphocytes. Human peripheral blood lymphocytes were obtained from healthy volunteers, purified by Ficoll-Hypaque flotation (9), washed twice in PBS, and viable cells were counted in eosin.

Stimulating Cells. Cells used for stimulation were treated with mitomycin C (subscript m) (10) and washed twice or they were X-irradiated (subscript x) (2,000 R at 250–300 R/min).

MLC Test. Triplicate cultures were set up with 1×10^5 human responding cells and 4×10^5 human or 1×10^6 mouse-stimulating cells in a flat-bottom well of a microtiter plate (Linbro Scientific Co., New Haven, Conn.) in 200 μl of medium RPMI 1640 (Gibco) supplemented with 10% (vol/vol) heat-inactivated human plasma (30 min at 56°C), penicillin (120 U/ml), and streptomycin (120 μg/ml). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Primary MLC cultures were harvested on day 5 after 20-h labeling with 2 μCi [^{3}H]thymidine (2 Ci/mmol, New England Nuclear, Boston, Mass.) added in 50 μl of RPMI 1640. Secondary MLC cultures were harvested on day 2 after 6-h labeling with [^{3}H]thymidine.

Primed Lymphocytes. 10 million human responding cells were cultured with 1×10^7 human or 4×10^7 mouse-stimulating cells in 30 ml of the medium used for MLC in an upright Falcon no. 3013 flask (Falcon Plastics, Oxnard, Calif.). 25% of the medium was changed on days 3 and 6 or 7. On day 9 or 10 the surviving cells were scraped off the bottom of the flask with a rubber policeman, resuspended in fresh medium, and restimulated as described under MLC test.

Mitogen Stimulation. 1×10^5 responding cells were cultured in 200-μl medium as described for the MLC test either with 3 μl phytohemagglutinin (PHA)-M stock (Difco Laboratories, Detroit, Mich.) and harvested on day 3 after 20 h of labeling with [^{3}H]thymidine or with 2 μg/ml pokeweed mitogen (PWM) (Gibco) and harvested on day 6 after 20 h of labeling.

Rosette Formation. E rosettes were formed by mixing 3×10^5 human lymphocytes in 100 μl RPMI 1640 with 100 μl 0.5% SRBC in PBS and 20 μl human AB serum (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) absorbed with sheep red blood cells (SRBC). The mixture

was centrifuged 5 min at 70 *g* and incubated overnight in the cold. All assays were set up in duplicate and counted blind. All leukocytes were counted, including clumps, and cells with three or more SRBC attached were considered rosette forming.

Cell Separation. Rosette formation was carried out as above with fivefold increased volumes in Falcon no. 2057 tubes. The mixtures were incubated only 2 h in the cold, and the contents of six tubes were separated on 3 ml Ficoll-Hypaque by centrifugation for 30 min at 400 *g*. The cells at the interface were removed and washed twice. The cells in the pellet were treated for 2 min at 37°C with a Tris-buffered 0.83% solution of NH_4Cl to remove SRBC and washed twice. Cells nonadherent to nylon wool columns were prepared as described (11). Cells nonadherent to plastic were prepared by three 1-h incubations at 37°C of 15–20 × 10^6 lymphocytes in RPMI 1640 with 25% (for human) or 5% (for mouse) heat-inactivated fetal calf serum on 60-mm plastic Petri dishes.

Presentation of the Data. Results are either presented directly as mean counts per minute (cpm) of [^{3}H]thymidine incorporated in triplicate cultures or they are converted to the percent of specific restimulation. This expresses the response of primed lymphocytes to a given stimulus as a percentage of the secondary response to the specific primary stimulator after correction for the response to fresh medium alone according to the formula:

$$\%\text{ of specific restimulation} = \frac{\text{Mean cpm with experimental stimulus} - \text{mean cpm with medium}}{\text{Mean cpm with specific stimulus} - \text{mean cpm with medium}} \times 100$$

Restimulation with human lymphocytes from the donor of the responding cells often led to a response higher than to medium alone. However, since the response to medium alone was, in most cases, comparable to the response to some third party stimulating mouse cells, the secondary responses presented here (except in Fig. 1) have all been corrected by subtraction of response to medium alone.

Controls to show that all stimulating cells stimulate specifically sensitized responders, and only those were always carried out; no data are shown unless these controls were satisfactory in that experiment.

Terminology. Randomly chosen human donors are represented by the letters H or G or by their initials, and mouse stimulators are represented by the letters M and N or by their strain designation.

Six regions and subregions of the *H-2* complex will be considered in this paper: the *K*, *I*, *S*, and *D* regions and the *I-A*, *I-B*, and *I-C* subregions of the *I*-region, which will be abbreviated as *A*, *B*, and *C* in the tables.

Results

The Xenogeneic PLT Technique. A primary xenogeneic human-mouse MLC response is detectable on day 3 and peaks on day 6 or 7 (12). If the sensitized lymphocytes from a primary culture are restimulated on day 9 with the specific primary stimulator, a rapid secondary response follows, usually peaking on day 3 (Fig. 1). Since in one case (see Fig. 1, panel a) the response already reached its maximum on day 2, this was chosen as the standard day of assay.

Stimulation by H-2 and M1s Loci. Fig. 1 shows that *H-2* genes are of major importance for restimulation of primed human lymphocytes. Thus, in panel (a) lymphocytes primed by DBA/2 *(H-2^d)* are restimulated to about the same response by DBA/2 and by B10.D2 *(H-2^d)*, which presents the same *H-2* haplotype on a different background. Panel (b) indicates that the non-H-2 genes are only of minor importance, since responders primed with B10.A(5R) *(H-2^{15})* give only slightly higher responses to B10.S *(H-2^s)*, which has the same background, than to A.SW *(H-2^s)*.

The *M1s* locus (13), which is not linked to *H-2*, in some *H-2*-identical combinations leads to strong primary allogeneic MLC responses. C57BL/10 and its

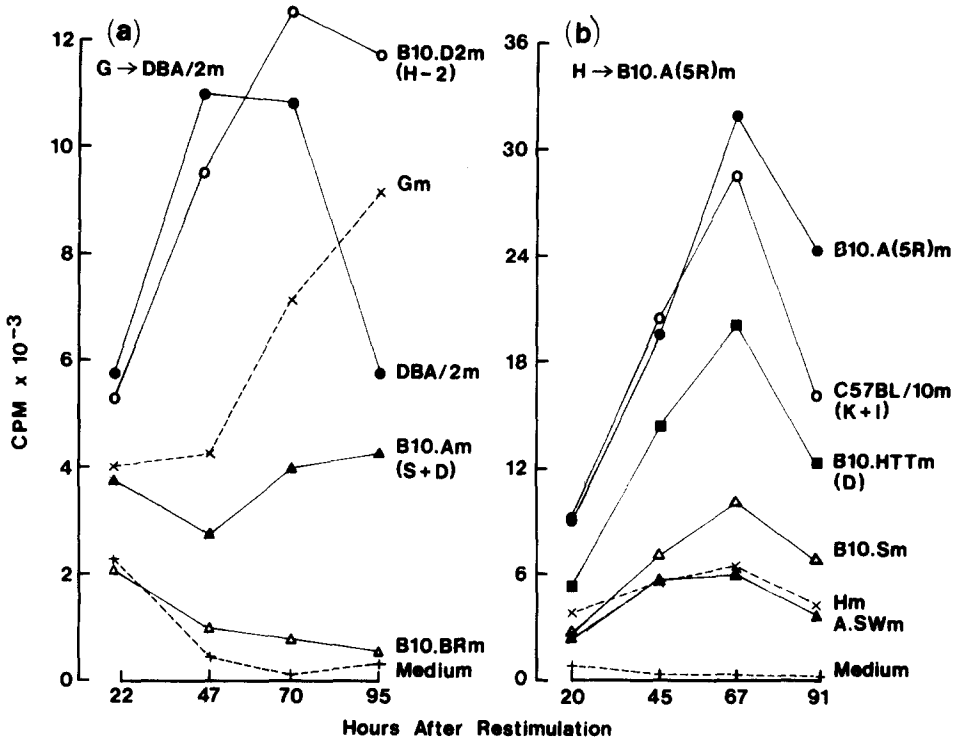

FIG. 1. Kinetics of secondary xenogeneic MLC response by human lymphocytes primed with mouse lymphocytes in vitro: results from two experiments with different human responders. The strains used for sensitization are indicated in the upper left hand corner of each panel and the donors of the restimulating cells next to the graphs. In parentheses are shown the regions of *H-2* these strains share with the specific stimulator. G_m (a) and H_m (b) are autologous, mitomycin-treated cells.

congenic partners all carry a very weak or nonstimulatory allele of this system, $M1s^b$. The strongest allele (as defined in a primary allogeneic MLC) of the *M1s* system, $M1s^a$, is shared by strains AKR *(H-2^k)*, DBA/1 *(H-2^q)*, and DBA/2 *(H-2^d)*. To assay the importance of non-H-2 antigens in general and the *M1s* locus in particular in xenogeneic MLC, we sensitized human lymphocytes to each of these strains and to their *H-2*-identical, B10 congenic partners B10.BR *(H-2^k)*, B10.G *(H-2^q)*, and B10.D2 *(H-2^d)*. The sensitized lymphocytes were restimulated with cells from each of the six strains. For example cells primed to AKR were tested for their response upon restimulation with a strain that shared *H-2* (B10.BR), $M1s^a$ (DBA/1 or DBA/2), or no known strong alloantigens (B10.G and B10.D2) with the initial stimulus.

It is clear from Table II that the secondary response was mainly stimulated by *H-2* antigens, and this response was not significantly augmented when primary and secondary stimulators were identical for the B10 background or shared the $M1s^a$ allele. When primary and secondary stimulator differed for *H-2*, it was sometimes possible to detect a response to an antigen(s) shared by AKR, DBA/1, and DBA/2, likely to be $M1s^a$. This is further supported by the lack of stimulation by the B10 background, which carries a silent *M1s* allele. The response to

TABLE II

Relative Importance of Mls and H-2 for Restimulation of Primed Human Lymphocytes. Summary of Two Consecutive Experiments with a Total of Four Responders

Antigens shared by 1° and 2° stimulator	No. of combinations	CPM* after restimulation Mean ± SE	P‡
Mls^a + H-2	10	22,898 ± 3,329	
"B10" + H-2	11	18,186 ± 2,893	NS
H-2	21	19,606 ± 1,641	NS
Mls^a	19	4,874 ± 977	
"B10"	22	1,661 ± 327	<0.01
None	41	2,147 ± 383	NS
Medium alone	21	1,181 ± 105	

* After subtraction of cpm with medium alone.

‡ Wilcoxon's signed rank test. NS, not significant ($P > 0.05$).

medium alone was arbitrarily chosen as a background, and it is impossible to determine whether all mice share a species-specific antigen that induces a minor response of the primed lymphocytes. It is clear, however, that this response constitutes at most 15% of the specific response to *H-2*-controlled antigens.

H-2 Control of Xenogeneic MLC Stimulation. By choosing primary and secondary stimulators that share only a single region within *H-2*, we further localized the determinants that activate human lymphocytes in MLC. Results of repeated tests of some selected critical strain combinations are shown in Table III. All results with 65 strain combinations tested with three different human responders in two experiments are summarized in Table IV. These experiments (illustrated in Table III) were chosen because they included most strain combinations and because the third party responses were particularly low, allowing better discrimination between the responses to various regions of *H-2*. However, all other experiments followed the same pattern.

No Shared H-2 Region. Table IV shows that the average restimulation by third party cells that share no *H-2* regions with the primary stimulator is only 5% of specific restimulation. This again attests to the minimal restimulation by genes in the B10 background, and it shows that there is little if any cross-reactivity of the H-2 determinants. Therefore, when a given combination shares only one *H-2* region, a positive response is more likely to be caused directly by antigens controlled by that region than by cross-reactivities of other regions in *H-2*.

H-2 K. The critical combination of primary and secondary stimulator is AQR and B10.G, which in most cases leads to a moderate secondary response.

H-2 I-A. Because the PLT test requires strains that differ everywhere except in the region under study, it allows independent assessment of the *H-2 I-A*

TABLE III

Response after Restimulation of Primed Human Lymphocytes in Some Critical Combinations of Primary and Secondary Stimulator

1° stimulator	2° stimulator	*H-2* regions shared by 1° and 2° stimulator						Mean cpm (after subtraction of cpm with medium alone) after restimulation		
		K	*A*	*B*	*C*	*S*	*D*	No. 187* M. G.	No. 190 K. B.	No. 190 C. U.
B10.A(4R)	B10.A(4R)	+	+	+	+	+	+	12,146	12,382	21,997
k kbb b b‡	B10.AKM	+	+					18,860	4,497	14,881
	AQR		+					21,347	2,196	7,370
	B10.A(5R)			+				92	50	4,520
	C57BL/10			+	+	+	+	1,161	1,087	2,002
	B10.D2							−749	−827	1,062
	B10.G							−605	398	1,099
	Medium							2,032	1,736	1,189
	Hm§							15,186	10,357	7,661
AQR	AQR	+	+	+	+	+	+	48,343	20,060	22,962
q kkd d d‡	B10.G	+						13,367	4,390	8,912
	B10.A(4R)		+					17,972	8,725	10,451
	B10.AKM		+	+				25,684	15,716	14,814
	B10.D2				+	+	+	6,889	2,201	4,831
	C57BL/10							926	21	2,020
	Medium							2,859	1,977	1,297
	Hm§							11,717	20,757	4,737
C57BL/10	C57BL/10	+	+	+	+	+	+	21,001	26,544	17,922
b bbb b b‡	B10.A(5R)	+	+	+				15,079	23,686	21,541
	B10.A(4R)			+	+	+	+	5,518	2,652	1,518
	B10.A(2R)						+	4,834	1,478	1,891
	B10.AKM							279	1,016	1,184
	B10.D2							−467	453	471
	B10.S(7R)							1,567	NT‖	NT
	Medium							3,800	1,261	532
	Hm§							16,320	20,116	6,033
B10.D2	B10.D2	+	+	+	+	+	+	22,983	8,777	6,608
d ddd d d‡	B10.A(5R)				+	+	+	6,547	1,674	2,536
	B10.A(2R)				+	+		−9	−184	1,462
	B10.S(7R)						+	7,204	NT	NT
	C57BL/10							1,417	−345	382
	B10.AKM							1,628	322	1,242
	Medium							2,887	1,639	913
	Hm§							18,486	13,386	4,536

* Experiment number and responder.

‡ Lower case letters indicate origin of the *H-2* regions *K*, *I-A*, *I-B*, *I-C*, *S*, and *D* of the primary stimulator.

§ Hm, 4×10^5 cells autologous to responder. Counts with medium alone have not been subtracted from these responses.

‖ NT, not tested.

subregion, which has only been tested in conjunction with *H-2 K* or *H-2 I-B* in primary allogeneic mouse MLC. The critical combination is AQR and B10.A(4R). The *H-2 I-A* subregion consistently induces a positive PLT response, the strongest response induced by a single *H-2* region or subregion. This is the first direct demonstration of an LD determinant in the *I-A* subregion.

H-2 I-B. The critical strains are B10.A(4R) and B10.A(5), which share a *b* allele in this subregion. It did not lead to any detectable restimulation.

H-2 I-C + H-2 S. The combinations tested were B10.A(2R)-B10.D2 or

TABLE IV
Genetic Control of Xenogeneic MLC

Region of H-2 shared						Percent of specific restimulation			Allele
K	*A*	*B*	*C*	*S*	*D*	Mean	Range	*n**	
None						5.0	0–58	107(33)	*b,d,k,q,s*
		+				5.0	0–21	6(1)	*b*
			+	+		12.9	0–43	11(3)	*d,k*
		+	+	+	+	11.2	5–26	6(1)	*b*
					+	20.2	0–44	27(12)	*b,d,q*
			+	+	+	29.4	5–97	18(3)	*d*
+						32.5	0–97	6(1)	*q*
+					+	57.0	8–151	4(1)	*q + d*
+	+					65.5	24–97	6(1)	*k*
+	+	+				85.1	45–121	12(3)	*b,k,s*
+	+				+	80.0	37–103	5(1)	*k + b*
+	+	+	+	+		62.4	27–97	5(2)	*q,s*
	+					40.6	18–101	6(1)	*k*
	+	+				85.9	40–183	6(1)	*k*
	+	+	+	+		84.0	50–154	5(1)	*k + d*

* Total number of combinations of responder and 1° stimulator and 2° stimulator tested. In parenthesis is shown the number of different strain combinations (M-N) used; some were tested both ways (i.e., priming with M and restimulating with N and vice versa), some only one way, and some with only one responder.

B10.A(5R) and B10.AKM-B10.HTT. The response was very weak, and we therefore did not test each region separately.

H-2 D. Several combinations were available to test the *H-2 D* region in this xenogeneic system. Some combinations, such as B10.G-B10.AKM and B10.S(7R)-B10.D2, tested only the *D* region, while others may have included *H-2 G*, for example C57BL/10-B10.A(2R). The average response to restimulation with *H-2 D* was moderate, not quite as strong as to *H-2 K*.

More Than One H-2 Region Shared. Restimulation by more than one *H-2* region is often comparable to the sum of the effects of the individual regions; thus, *K* (32%) + *D* (20%) together give 57% of specific restimulation, and *K* (32%) + *I-A* (41%) together give 66%. There are two exceptions to this pattern: the *b* alleles of the regions *I-B* through *D* together give less stimulation than *H-2 D* alone or *I-C* + *S* alone, and *I-A* together with the very weakly stimulatory *I-B* and *I-C* + *S* regions causes much higher responses than the sum of the individual regions (Table IV). Both exceptions could be ascribed to differences between haplotypes or to epistatic interactions between alleles of separate loci. It is possible that the restimulation caused by the *I-A* region alone in the combination 4R-AQR is exceptionally low, or the high response to a whole *I* region could be due to an *LD* locus between the *I-B* and *I-C* subregions.[2]

In summary, the *K*, *I-A*, and *D* regions cause significant stimulation of human lymphocytes. The stimulation by the *I* region alone is significantly higher than by the *K* or *K* + *D* regions, and the *K* + *I* regions together do not

[2] Lindahl, K. F. and F. H. Bach. Manuscript in preparation.

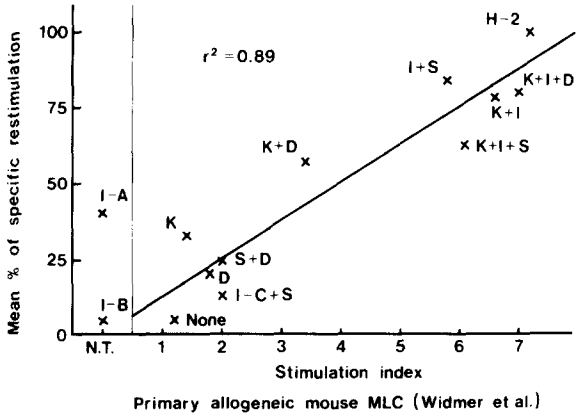

FIG. 2. Comparison of the relative strength of various regions of the *H-2* complex in stimulation of allogeneic and xenogeneic MLC responses. Next to each point is indicated the region(s) that differed in the allogeneic MLC test or was shared in the xenogeneic PLT test. The *I-A* and *I-B* subregions were not tested individually in the study by Widmer et al. (14); otherwise, the average stimulation index obtained in that study was used. A stimulation index of 1.0 means no MLC response. The line was determined by linear regression analysis (percent of specific restimulation = 0.05 + 12.5 × stimulation index [$r^2 = 0.89$]).

stimulate significantly more than the *I* region alone, showing that the strongest xenogeneic LD determinants are located in the *I* region, more specifically in the *I-A* subregion.

Correlation of Xenogeneic PLT with Allogeneic MLC. Each region of *H-2* seemed to play a similar role in restimulation of primed human lymphocytes and in allogeneic MLC. This was further verified by comparing the human responses summarized in Table IV with the average stimulation indexes found in allogeneic MLC (Fig. 2). As shown, there is a highly significant, linear correlation between responses by mouse and by human lymphocytes. The strains tested by Widmer et al. (14) are the same strains used here. We have also compared our results to those of other groups, and in each case xenogeneic and allogeneic responses correlate very well: Spearman's rank correlation coefficient $r_s = 0.86$ ($n = 7, P < 0.05$) for Dorf et al. (15) and $r_s = 0.90$ ($n = 9, P < 0.01$) for Meo et al. (16).

The most likely explanation of this correlation is that human lymphocytes are stimulated in MLC by the same H-2 determinants that stimulate allogeneic mouse lymphocytes.

Cellular Requirements of Xenogeneic MLC. Since the stimulating antigens appear to be identical in allogeneic and xenogeneic MLC reactions, it was of interest to establish whether the homology between the reactions extends to the cellular requirements. Table V shows that adherent cells, which can be removed by incubation on plastic, are required both for an allogeneic and a xenogeneic MLC response. These cells do not need to divide, since the response can be reconstituted by addition of a small number of mitomycin C treated, unseparated cells. While both autologous and allogeneic human macrophages will reconstitute the response of purified lymphocytes (Table V and no. 17–19), the adherent cells of the stimulating mouse spleen cell population can not support human lymphocytes.

TABLE V
Need for Adherent Cells in Allogeneic and Xenogeneic MLC

Responding cells	Response (mean cpm ± SD) to stimulating cells*		
	Hpur‡$_m$	Gpur‡$_m$	M_m
H	504 ± 116	19,913 ± 2,954	8,175 ± 931
Hpur§	475 ± 379	4,719 ± 1,790	2,137 ± 495
Hpur + 5% H_m§	358 ± 300	13,508 ± 1,861	10,247 ± 1,663
Hpur + 5% G_m	3,036 ± 553	19,473 ± 2,991	

* H, human responder; G, allogeneic human; M, mouse; all mitomycin treated.
‡ Purified of adherent cells by three sequential incubations in plastic Petri dishes.
§ Mixture of 1×10^5 purified responding cells and 5×10^3 unfractionated, mitomycin-treated cells.

TABLE VI
Response of Fractionated Human Lymphocytes to Allogeneic and Xenogeneic Lymphocytes and Mitogens

	Day of assay	H unseparated*	H interface* + 10% H_m	H pellet* + 10% H_m	H nonadherent* + 10% H_m
E-RFC (%)	0	54 ± 2.8‡	4 ± 4.2‡	90 ± 1.4‡	78 ± 13.4‡
Stimulus					
H_m	5	1,538 ± 138§	1,725 ± 304	1,103 ± 179	973 ± 453
G_m	5	13,419 ± 1,596	8,543 ± 1,610	11,402 ± 107	9,791 ± 2,349
M_m	5	20,842 ± 6,536	1,433 ± 353	24,102 ± 1,149	10,935 ± 3,289
N_m	5	18,039 ± 1,959	2,651 ± 342	19,417 ± 2,572	10,521 ± 2,524
PHA	3	103,725 ± 5,221	6,497 ± 472	117,129 ± 5,706	92,865 ± 6,375
PWM	5	105,693 ± 13,701	13,100 ± 2,065‡	38,752 ± 2,397	16,768 ± 1,824

* In each well was 1×10^5 responding lymphocytes from donor H. The cells recovered from the interface and the pellet after separation of E rosettes on a Ficoll-Hypaque gradient and the cells that did not adhere to nylon wool were supplemented with 1×10^4 unfractionated, mitomycin-treated autologous cells.
‡ Mean and SD of duplicates.
§ Mean cpm [^{3}H]thymidine incorporated ± SD of triplicate cultures.

Human peripheral blood lymphocytes can be separated by E rosetting into a fraction enriched for B lymphocytes and macrophages (the interface from a Ficoll-Hypaque gradient) and a fraction enriched for T lymphocytes (the pellet) (20). One can also enrich for T lymphocytes by passing the cell suspension over a nylon wool column (11). The ability of such enriched cell populations from donor H to respond to mouse (M_m and N_m) and allogeneic human (G_m) lymphocytes and to mitogens was tested as shown in Table VI. Since both the pellet fraction and the cells that did not adhere to nylon wool were partially depleted of adherent cells necessary for the xenogeneic MLC response, we supplemented the enriched cell populations with 10% mitomycin C treated, unseparated, autologous cells.

Both cell populations enriched for T lymphocytes gave responses within the normal range to xenogeneic and allogeneic lymphocytes and to the T-cell mitogen, PHA, and they gave clearly positive, albeit decreased, responses to PWM, which is a combined T- and B-cell mitogen (21). The B-cell fraction recovered from the interface gave only a minimal response to PHA, did not respond significantly to mouse lymphocytes, but could still respond to PWM and to allogeneic lymphocytes. We interpret the response of the B-cell fraction to allogeneic lymphocytes as an example of "recoil-activation", which is known from studies of allogeneic mouse MLC. Thus mouse B lymphocytes can prolifer-

ate in MLC provided that they present antigens foreign to the "stimulator" and that the stimulating cells contain T cells (22–24). It is interesting to note that the mouse stimulating cells do not "recoil-activate" the human responder cells.

In a total of eight experiments, the median xenogeneic MLC response of the B-cell fraction, which had from 0 to 16% cells forming rosettes with sheep erythrocytes (E-RFC), was 19.5% of the response of unseparated cells and only significant in three out of the eight cases. Thus T cells are required for a xenogeneic MLC response. The response of the T-cell fractions (61–90% E-RFC) was 135% of that of unseparated cells for the pellet fraction and 116% of normal for the nylon wool purified cells. After 9 days of culture, a mean of 70% E-RFC were found in both allogeneic and xenogeneic cultures, indicating that the majority of the proliferating cells are T cells (12). Xenogeneic MLC responses are thus mediated by T lymphocytes and require adherent cells as do allogeneic MLC responses, but in the human-mouse system, both T cells and adherent cells must come from the human responder.

Discussion

In this paper we have used "primed LD typing" (2) to determine which antigens of mouse lymphocytes activate human lymphocytes in mixed lymphocyte culture. Human lymphocytes sensitized to cells of one mouse strain gave a strong response upon restimulation with cells from the same strain or from an H-2-identical strain. Non-H-2 antigens carried in the C57BL/10 background accounted for less than 15% of specific restimulation. The strongest allele of the *Mls* locus, Mls^a, induced relatively weak and inconsistent restimulation amounting to a mean of 25% of an H-2 response. The mouse is so far the only species where a strong lymphocyte-activating determinant not linked to the MHC has been described. The Mls antigens do not have all the properties of an H-2 LD determinant, in particular they induce only very weak graft-versus-host reactions in vivo (25).

Since the PLT approach requires strains that differ at all *LD* loci except for the genetic region under study, we were able to study the role of each *H-2* region and subregion separately. In particular, we could directly demonstrate a strong LD determinant in the *I-A* subregion, which has only been tested in combination with the *K* or *I-B* regions in allogeneic MLC. The *K* and *D* regions caused moderate and the *I-C* + *S* regions weak restimulation. As Fig. 2 shows, the strength of the various *H-2* regions in allogeneic and in xenogeneic MLC correlates very well, suggesting that human and mouse lymphocytes respond to the same LD determinants of the mouse. In accordance with these findings, Howard[3] recently observed that rat lymphocytes selected in vitro for response to a given mouse strain were restimulated more by mouse lymphocytes sharing the *K* end of *H-2* with the primary stimulator than by cells sharing the *D* end.

The restimulation system was remarkably specific, the cross-reactivity of mouse LD determinants amounting to less than 10–15% of specific responses. It is possible that the human T cells recognize species-specific antigens of mouse lymphocytes, but these either play a very minor role or they are only recognized as they interact with or are modified by H-2. A similar absence of cross-

[3] Howard, J. C. Personal communication.

reactivity has been observed in rat-mouse restimulation systems (26, and footnote 3). This noncross-reactivity contrasts with studies using suicide techniques. When human lymphocytes responding to lymphocytes of one dog were eliminated, the remaining cells gave a reduced response to stimulation with cells from another dog (27), similar observations were made in human-mouse, lamb-human, and lamb-mouse systems (28, 29). The reduction of the xenogeneic responses in these systems could be due to actual sharing of MHC LD determinants by the primary and secondary stimulator, but it might also be a nonspecific phenomenon.

We have recently described how human lymphocytes sensitized in vitro against mouse spleen cells become specifically cytotoxic against mouse target cells sharing the *H-2 K* or *H-2 D* region with the stimulating cells (30). Thus, cells sensitized to strain A will kill target cells of strain B, which share only the *K* and *D* regions of *H-2* with A, as well as effector cells specifically sensitized to B do; but the same effector cells will not kill target cells of strain C, which share the whole *I* and *S* region with strain A. Differential cytotoxicity against non-H-2 antigens was not detectable in this system (12), and species-specific antigens played a very minor role, if any. Thus, the specificity of xenogeneic cytotoxic effector cells parallels that of allogeneic effector cells (31–34). The absence of detectable cytotoxicity against *I*-region determinants, which can be found in sensitive allogeneic systems (35–38), may be due to the relative inefficiency of the xenogeneic cytotoxicity system.

Human responder cells thus reacted in the MLC and CML assays to the same antigenic determinants of mouse-stimulating cells in the same manner as allogeneic mouse responder cells do, and the same classes of human cells were involved in the xenogeneic response as in an allogeneic MLC. Adherent cells (17, 18) and T lymphocytes (39) were required, and the majority of the proliferating cells were T cells. It is interesting that the mouse stimulator cells, in contrast to allogeneic stimulator cells, could provide neither the adherent cell nor the T-cell function. This may be a general phenomenon. Greineder and Rosenthal (40) recently observed that mouse macrophages do not support the proliferative response of guinea pig lymphocytes to oxidation with periodate, whereas guinea pig macrophages do. We suggest that the relative weakness of graft-versus-host reactions in xenogeneic combinations (41–43) may be due to a failure of cells from different species to interact rather than to a lack of cells in the inoculum reactive to the xenogeneic histocompatibility antigens. Such interactions between graft and host cells may well be necessary to activate secondary reactions, which may even involve host cells, that potentiate the graft-versus-host reaction and lead to development of the late symptoms of graft-versus-host disease. The greater strength of alloaggression reactions might thus be due to secondary potentiating mechanisms, whereas the fundamental immunological nature of reactions to allogeneic and xenogeneic lymphocytes appears to be the same, a reaction of T lymphocytes to foreign MHC LD and SD determinants leading to proliferation and cytotoxicity.

Summary

The nature of the antigens stimulating xenogeneic lymphocytes was studied using "primed LD typing." Human lymphocytes were sensitized in vitro against

mouse spleen cells and restimulated with spleen cells of mouse strains sharing non-H-2 antigens or various regions of *H-2* with the initial stimulating strain. The largest thymidine uptake was caused by restimulation with cells from the specific primary stimulator or an *H-2*-identical strain. Species-specific antigens or strain-specific antigens carried in the C57BL/10 background account for less than 15% of the total stimulation; a non-H-2 antigen associated with the Mls^a genotype caused moderate restimulation, amounting to 25% of the average *H-2* response. Within *H-2*, the strongest restimulation was caused by antigens controlled by the *I-A* subregion; the *K* and *D* regions caused moderate, the *I-C* and *S* regions very weak, and the *I-B* subregion no restimulation. Thus, the genetic control of antigens stimulating xenogeneic and allogeneic MLC responses appears identical. Like an allogeneic MLC reaction, the xenogeneic MLC response requires T cells and adherent cells, but in the human-mouse MLC, both cell types must come from the human responder; the majority of the proliferating cells are T cells. It is suggested that allograft and xenograft reactions are fundamentally identical processes, and that the relative vigor of alloaggression may be explained by secondary potentiating mechanisms depending on species-specific interactions between aggressor and target cells.

We thank Dr. J. Zarling for materials and advice for E rosetting, Mr. M. J. Sheehy for suggesting the PLT approach, Ms. J. Witson and Ms. G. Gordon for technical assistance, and Ms. S. Anderson and Ms. N. Van Der Puy for secretarial help.

Received for publication 29 March 1976.

References

1. Widmer, M. B., and F. H. Bach. 1972. Allogeneic and xenogeneic response in mixed leukocyte cultures. *J. Exp. Med.* 135:1204.
2. Sheehy, M. J., P. M. Sondel, M. L. Bach, R. Wank, and F. H. Bach. 1975. HL-A LD typing: a rapid assay using primed lymphocytes. *Science (Wash. D. C.).* 188:1308.
3. Häyry, P., and L. Andersson. 1974. Generation of T memory cells in one-way mixed lymphocyte culture. II. Anamnestic responses of 'secondary' lymphocytes. *Scand. J. Immunol.* 3:823.
4. Howard, J. C., and D. B. Wilson. 1974. Specific positive selection of lymphocytes reactive to strong histocompatibility antigens. *J. Exp. Med.* 140:660.
5. Sheehy, M. J., P. M. Sondel, F. H. Bach, M. L. Sopori, and M. L. Bach. 1975. Rapid detection of LD determinants: the PLT assay. *In* Histocompatibility Testing 1975. F. Kissmeyer-Nielsen, editor. Munksgaard, Copenhagen. 569.
6. Mawas, C. E., D. Charmot, and M. Sasportes. 1975. Secondary response of in vitro primed human lymphocytes to allogeneic cells. I. Role of HL-A antigens and mixed lymphocyte reaction stimulating determinants in secondary in vitro proliferative response. *Immunogenetics.* 2:449.
7. Bradley, B. A., M. J. Sheehy, J. Keuning, A. Termijtelen, D. Franks, and J. J. van Rood. 1976. Phenotyping of HLA-D region products by negative and positive mixed lymphocyte reactions. *Immunogenetics.* In press.
8. Peck, A. B., and F. H. Bach. 1973. A miniaturized mouse mixed leukocyte culture in serum-free and mouse serum supplemented media. *J. Immunol. Methods.* 3:147.
9. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77.

10. Bach, F. H., and N. K. Voynow. 1966. One-way stimulation in mixed leukocyte cultures. *Science (Wash. D. C.)*. 153:545.
11. Greaves, M. F., and B. Brown. 1974. Purification of human T and B lymphocytes. *J. Immunol.* 112:420.
12. Lindahl, K. F. 1975. Specificity of xenograft reactions in vitro. Ph. D. Thesis. University of Wisconsin.
13. Festenstein, H. 1973. Immunogenetic and biological aspects of *in vitro* lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.* 15:62.
14. Widmer, M. B., C. Omodei-Zorini, M. L. Bach, F. H. Bach, and J. Klein. 1973. Importance of different regions of H-2 for MLC stimulation. *Tissue Antigens*. 3:309.
15. Dorf, M. E., J. M. D. Plate, J. H. Stimpfling, and B. Benacerraf. 1975. Characterization of immune response and mixed lymphocyte reactions in selected intra-H-2 recombinant strains. *J. Immunol.* 114:602.
16. Meo, T., G. Vives, A. M. Rijnbeek, V. C. Miggiano, M. Nabholz, and D. C. Shreffler. 1973. A bipartite interpretation and tentative mapping of H-2-associated MLR determinants in the mouse. *Transplant. Proc.* 5:1339.
17. Alter, B. J., and F. H. Bach. 1970. Lymphocyte reactivity in vitro. I. Cellular reconstitution of purified lymphocyte response. *Cell. Immunol.* 1:207.
18. Rode, H. N., and J. Gordon. 1970. The mixed leukocyte culture: a three component system. *J. Immunol.* 104:1453.
19. Rode, H. N., and J. Gordon. 1974. Macrophages in the mixed leukocyte culture reaction (MLC). *Cell. Immunol.* 13:87.
20. Wybran, J., S. Chantler, and H. H. Fudenberg. 1973. Isolation of normal T cells in chronic lymphatic leukaemia. *Lancet.* 1:126.
21. Greaves, M. F., and G. Janessy. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. *Transplant. Rev.* 11:87.
22. Harrison, M. R., and W. E. Paul. 1973. Stimulus-response in the mixed lymphocyte reaction. *J. Exp. Med.* 138:1602.
23. Croy, B. A., and D. Osoba. 1973. Nude mice and the mixed leukocyte reaction. *Transplant. Proc.* 5:1721.
24. Piquet, P-F, H. K. Dewey, and P. Vassalli. 1975. Study of the cells proliferating in parent versus F_1 hybrid mixed lymphocyte culture. *J. Exp. Med.* 141:775.
25. Abbasi, K., P. Demant, H. Festenstein, J. Holmes, B. Huber, and M. Rychlikova. 1973. Mouse mixed lymphocyte reactions and cell-mediated lympholysis: genetic control and relevance to antigenic strength. *Transplant. Proc.* 5:1329.
26. Hollander, N., H. Ginsberg, and M. Feldman. 1974. In vitro generation of memory lymphocytes to transplantation antigens. *J. Exp. Med.* 140:1057.
27. Hirschberg, H., B. Rankin, and E. Thorsby. 1974. Specificity of human lymphocytes proliferating in response to allogeneic and xenogeneic cells in vitro. *Transplantation (Baltimore)*. 17:593.
28. Asantila, T., J. Vahala, and P. Toivanen. 1974. Response of human fetal lymphocytes in xenogeneic mixed leukocyte culture: phylogenetic and ontogenetic aspects. *Immunogenetics*. 1:272.
29. Toivanen, P., and T. Asantila. 1974. Ontogeny of allogeneic and xenogeneic cell recognition. II. Studies on a fetal lamb. *Scand. J. Immunol.* 3:891.
30. Lindahl, K. F., and F. H. Bach. 1975. Human lymphocytes recognize mouse alloantigens. *Nature (Lond.)*. 254:607.
31. Alter, B. J., D. J. Schendel, M. L. Bach, F. H. Bach, J. Klein, and J. H. Stimpfling. 1973. Cell-mediated lympholysis. Importance of serologically defined *H-2* regions. *J. Exp. Med.* 137:1303.
32. Schendel, D. J., and F. H. Bach. 1974. Genetic control of cell-mediated lympholysis in mouse. *J. Exp. Med.* 140:1534.

33. Nabholz, M., J. Vives, H. Young, T. Meo, V. Miggiano, A. Rijnbeek, and D. Shreffler. 1974. Cell-mediated cell lysis *in vitro*: genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* 4:378.
34. Peck, A. B., B. J. Alter, and K. F. Lindahl. 1976. Specificity in T cell mediated lympholysis: identical genetic control of the proliferative and effector phases of allogeneic and xenogeneic reactions. *Transplant Rev.* 29:189.
35. Peck, A. B., and F. H. Bach. 1975. Mouse cell-mediated lympholysis assay in serum-free and mouse serum-supplemented media: culture conditions and genetic factors. *Scand. J. Immunol.* 4:53.
36. Wagner, H., D. Götze, L. Ptschelinzew, and M. Röllinghoff. 1975. Induction of cytotoxic T lymphocytes against *I*-region-coded determinants: in vitro evidence for a third histocompatibility locus in the mouse. *J. Exp. Med.* 142:1477.
37. Nabholz, M., H. Young, A. Rynbeek, R. Boccardo, C. S. David, T. Meo, V. Miggiano, and D. C. Shreffler. 1975. I-region-associated determinants: expression on mitogen-stimulated lymphocytes and detection by cytotoxic T cells. *Eur. J. Immunol.* 5:594.
38. Schendel, D. J., and F. H. Bach. 1975. H-2 and non-H-2 determinants in the genetic control of cell-mediated lympholysis. *Eur. J. Immunol.* 5:880.
39. Wigzell, H., and P. Häyry. 1974. Specific fractionation of immunocompetent cells. Application in the analysis of effector cells involved in cell mediated lysis. *Curr. Top. Microbiol. Immunol.* 67:1.
40. Greineder, D. K., and A. S. Rosenthal. 1975. The requirement for macrophage-lymphocyte interaction in T lymphocyte proliferation induced by generation of aldehydes on cell membranes. *J. Immunol.* 115:932.
41. Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. *Acta Pathol. Microbiol. Scand.* 40:480.
42. Payne, L. N., and W. P. Jaffe. 1962. Graft-against-host reactions produced in chick embryos with blood from heterologous avian donors. *Transplant. Bull.* 30:20.
43. Lafferty, K. H., and M. A. S. Jones. 1969. Reactions of the graft versus host (GVH) type. *Aust. J. Exp. Biol. Med. Sci.* 47:17.