HUMAN MIXED LEUKOCYTE CULTURE: IDENTIFICATION OF THE PROLIFERATING LYMPHOCYTE SUBPOPULATION BY SEX CHROMOSOME MARKERS

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Although the mixed leukocyte cultures assay (MLC),[1] considered to represent an in vitro analogue of the recognition phase of allograft rejection, is widely used for evaluation of cellular immunoresponsiveness and for histocompatibility testing (1, 2), the cellular events leading to proliferation of a small fraction of the lymphocyte inoculum were only recently unveiled and are now understood in more detail. Of particular interest is the role of thymus-processed (T) and thymus-independent (B) lymphocytes in this system. Techniques have been developed for effective separation of human T and B lymphocytes (3), which now permit analyzation of the respective contribution of these lymphocyte subpopulations to stimulation and proliferation in MLC. We have recently demonstrated a strong stimulatory capacity of human B lymphocytes in MLC, whereas T cells only weakly stimulated allogeneic lymphocytes.[2] In other studies, we presented indirect evidence that proliferation in human MLCs is exclusively a function of T lymphocytes; no evidence for participation of B cells in the proliferative MLC response was obtained.[3]

Here we report the results of experiments which confirm the T-cell nature of the proliferating cells in human MLCs. Direct information on the differentiation of the proliferating cells was obtained using an in vitro chimeric system with T- and B-cell chromosome markers. This system was established by separation, and subsequent cross-recombination of T and B cells from HL-A genotypically identical, MLC-negative siblings of the opposite sex.

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[1] *Abbreviations used in this paper:* B, thymus-independent lymphocytes; MLC, mixed leukocyte cultures assay; N-SRBC, *Vibrio cholerae* neuraminidase-pretreated SRBC; PHA, phytohemagglutinin; SRBC, sheep erythrocytes; T, thymus-processed lymphocytes.

[2] H.-P. Lohrmann, L. Novikovs, and R. G. Graw. 1974. Stimulatory capacity of T and B lymphocytes in human mixed leukocyte cultures. *Nature (Lond.)*. In press.

[3] H.-P. Lohrmann, L. Novikovs, and R. G. Graw. 1974. Cellular interactions in the proliferative response of human T and B lymphocytes to phytomitogens and allogeneic cells. *J. Exp. Med.* **139**:1553.

Materials and Methods

Three pairs of HL-A genotypically identical siblings of the opposite sex with reciprocally negative stimulation in MLC were used as blood donors. Lymphocyte HL-A typing was performed by Paul I. Terasaki (Department of Surgery, U. C. L. A. School of Medicine, Los Angeles, Calif.) using standard microcytotoxicity methods (4). HL-A genotype identity between the siblings was established from the HL-A-typing results of other family members. Reciprocal nonreactivity of the siblings' lymphocytes in MLC was confirmed using techniques detailed below.

Separation of T and B Lymphocytes.—T and B lymphocytes from 60 to 100 ml of heparinized venous blood were purified as described previously.[3] Briefly, lymphocytes were separated by centrifugation over a Ficoll-Hypaque gradient (5). The distinctive feature of human T lymphocytes to form rosettes with unsensitized sheep erythrocytes (SRBC) under suitable conditions (6, 7) was used for separation of rosetting (=T) and nonrosetting (=B) lymphocytes. *Vibrio cholerae* neuraminidase-pretreated SRBC (N-SRBC) were used for rosette formation since they bind more firmly to human T cells than untreated SRBC (8, 9). After formation of T-lymphocyte rosettes, using the method described by Wybran and co-workers (10) with minor modifications, rosetted and nonrosetted cells were separated by centrifugation over a Ficoll-Hypaque gradient; owing to their greater specific gravity, rosetted lymphocytes form the pellet, whereas nonrosetted lymphocytes remain at the interface between the two layers. Morphologically, the pellet population consisted only of small lymphocytes (termed T cells). They were washed twice in Hanks' balanced salt solution, and resuspended in culture medium to the concentration needed. In order to minimize manipulations of lymphocytes, no efforts were made to lyse or remove the N-SRBC present in this preparation: orienting experiments showed that lymphocyte proliferation upon stimulation with phytomitogens or allogeneic lymphocytes was unaltered by addition of N-SRBC in this ratio. The interface cells were rerosetted and again centrifuged over a Ficoll-Hypaque layer, in an effort to remove any residual rosetting cells. The interface cell population obtained after this second procedure (termed "B cells" in this context) consisted of 80–85% small lymphocytes (B cells), 15–20% monocytes, and an occasional eosinophil or basophil granulocyte. These cells were resuspended in culture medium to the concentrations of small (B) lymphocytes needed.

Lymphocyte Culture Methods.—Lymphocyte culture methods have been described previously (11). For chromosome analyses, 5×10^5 rosetting (T) cells of one sibling were combined with 5×10^5 nonrosetting (B) lymphocytes of the sibling of opposite sex, in a total vol of 3 ml. One-way stimulation of these mixtures in MLC was achieved by adding 1×10^6 irradiated (2,500 rads) unseparated lymphocytes of an unrelated, HL-A-mismatched donor. Cultures were maintained for 7 days in a humidified 5% CO_2 air atmosphere, and the cells then processed for chromosome studies according to the method of Moorhead et al. (12).

Parallel cultures were established to assess the proliferation of the separated T and B cells after stimulation with phytohemagglutinin (PHA) and allogeneic lymphocytes from the uptake of tritiated thymidine [^{3}H]TdR into DNA. In these series, 2×10^5 T and/or B cells were stimulated by 2×10^5 allogeneic lymphocytes, or by 0.005 ml of a PHA stock solution prepared by dissolving the content of one vial of PHA-P (Difco Laboratories, Detroit, Michigan) in 5 ml of Eagle's minimum essential medium (MEM). PHA-stimulated cultures were harvested on day 3, and MLCs on day 7, as described previously (11), after [^{3}H]TdR (2 μCi) had been present for 4 h.

RESULTS

As determined by lymphocyte surface differentiation markers, the method utilized in the present studies for separation of rosetting and nonrosetting mononuclear cells is highly effective (reference 3 and footnote 3): preparations of nonrosetting cells contained less than 1% rosetting lymphocytes; and less

than 2% of the rosetting lymphocytes carried detectable amounts of surface immunoglobulins.

Effective separation of T and B cells was emphasized by the observation of a high [^{3}H]TdR uptake by T-cell preparations in response to PHA and allogeneic lymphocytes. In contrast, a negligible proliferative response of B-cell preparations was seen (Table I) which most likely has to be attributed to the few residual T cells contaminating the B-cell preparations, and does not represent true B-cell proliferation. When the unresponsive B cells were combined with T cells, the proliferative response was in excess of that expected from the proliferation of the two separated fractions (Table I), indicating a synergy between cells in the two populations in response to PHA and allogeneic lymphocytes. To determine whether B cells participated in the proliferative response to allogeneic lymphocytes in the presence of T cells, the following studies were performed.

TABLE I

*The Proliferative Response of Separated Human T and B Lymphocytes**

Stimulant	Responding cell type					
	Male T	Male B	Female T	Female B	Male T + female B	Male B + female T
Pair 1						
None	813	1,405	433	1,268	2,716	1,823
	±284	±156	±12	±137	±203	±201
PHA (0.003 ml)	157,108	2,303	102,583	2,793	240,565	208,570
	±27,874	±364	±14,266	±1,791	±33,504	±83,938
	$P < 0.001$				$P < 0.01$	
Pair 2						
None	629	1,764	587	1,560	2,471	2,117
	±48	±104	±53	±559	±156	±139
PHA (0.005 ml)	133,478	1,083	203,930	1,332	423,082	591,740
	±17,556	±51	±100,145	±871	±39,976	±22,892
	$P < 0.001$				$P < 0.001$	
Allograft cells (2×10^5)	120,352	2,303	165,682	5,491	163,420	201,667
	±20,503	±1,056	±15,330	±1,313	±4,381	±17,811
	$P < 0.01$				$P < 0.01$	
Pair 3						
None	701	5,249	647	4,173	5,027	2,108
	±362	±14	±128	±823	±486	±1,232
PHA (0.005 ml)	243,075	18,254	144,883	9,768	534,438	364,517
	±22,048	±144	±1,598	±766	±77,180	±31,423
	$P < 0.001$				$P < 0.001$	
Allograft cells (2×10^5)	94,804	9,148	33,924	9,005	130,700	68,924
	±5,099	±837	±9,075	±716	±11,277	±11,573
	$P < 0.01$				$P < 0.005$	

* T and B peripheral blood lymphocytes of three pairs of HL-A genotype identical, MLC-negative siblings of the opposite sex were prepared. 1-ml cultures, containing 2×10^5 T- and/or 2×10^5 B-responding lymphocytes, were set up in duplicate. PHA-stimulated cultures were harvested on day 3, and mixed leukocyte cultures on day 7. Stimulation was assessed from the uptake of tritiated thymidine into DNA, present during the last 4 h of culture. Indicated are the means ± one SD of [^{3}H]TdR uptake (dpm).

Direct evidence for the nature of the proliferating cells after stimulation of chimeric T + B-cell mixtures with allogeneic lymphocytes was obtained by karyotype analysis. Such analysis was based on the assumption that a male or female sex chromosome marker in a proliferating, metaphase-arrested cell indicated proliferation of the respective lymphocyte type (T or B) in the responding chimeric cell mixture. This assumption appears reasonable since highly purified T- and B-cell preparations were utilized.

Table II lists the frequency of sex chromosome markers among the proliferating cells in MLC, and attributes them to T- or B-cell origin. As can be seen, 86–100% (mean, 95.8 ± 5.6%; median 98%) of the proliferating cells carried T-cell chromosome markers. 174 of the analyzed 184 karyotypes were T-cell derived.

DISCUSSION

We have previously demonstrated[3] that interactions between different cell types after in vitro stimulation may lead to proliferation of B cells which by their nature are not responsive when they are isolated. Therefore, the demonstration that isolated T but not purified B cells proliferated upon stimulation with allogeneic lymphocytes (Table I) did not necessarily exclude proliferation of B cells in response to alloantigens when T + B-cell mixtures were stimulated. As shown in Table I, T + B-cell mixtures respond with significantly greater proliferation to allogeneic lymphocytes than either T or B cells alone. One possibility which was considered as the cause of this amplified cellular response was T-cell-mediated B-cell proliferation.

In order to determine the nature of the proliferating cells in alloantigen-stimulated T + B-cell mixtures, we utilized the reciprocal nonstimulation of

TABLE II

*Chromosome Markers in Proliferating Cells in MLC**

	Composition of responding cells			Sex chromosome pattern in proliferating cells (number of karyotypes)		Origin of proliferatig cells	
	Male		Female	Male	Female	T	B
						%	
Pair 1	T	+	B	52	2	96.3	3.7
	B	+	T	0	28	100	0
Pair 2	T	+	B	37	6	86	14
	B	+	T	0	25	100	0
Pair 3	T	+	B	26	2	93	7
	B	+	T	0	6	100	0

* 5×10^5 separated T cells from one sibling were combined with 5×10^5 isolated B cells from an HL-A genotype identical, MLC-negative sibling of opposite sex, and stimulated with 1×10^6 irradiated (2,500 rads) unseparated lymphocytes from an unrelated HL-A-mismatched donor. Cultures were harvested for karyotype analysis on day 7.

lymphocytes from genotypically HL-A identical siblings to create a chimeric system in vitro: by combining purified T lymphocytes of a first sibling with the purified B lymphocytes of the second sibling of the opposite sex, a nonstimulatory cell mixture was obtained in which T- and B-cell sex chromosome markers were permitted to determine the origin (male or female) and thus the nature (T or B) of any proliferating cell.

Karyotype analysis indicated that approximately 95% of the cells which proliferated in response to histoincompatible allogeneic lymphocytes were T-cell derived. Most likely, the residual 5% of the proliferating cells were not B cells, but were derived from T cells which contaminated the B-cell preparations. Thus, the demonstration of T-cell chromosome markers solely in the proliferating cells in chimeric T + B-cell mixtures (Table II) excludes secondary, T-cell-mediated B-cell triggering for human MLCs.

We have shown[3] that purified B cells as prepared in the present studies are capable of undergoing blastic transformation and proliferation under certain experimental conditions. Viability of the B cells is further demonstrated by their high spontaneous DNA synthesis, exceeding by far that of T cells (Table I). Furthermore, more than 95% of the B lymphocytes were viable as assessed by trypan blue exclusion. Finally, these B cells possess high stimulatory capacity in MLC,[2] a property which requires metabolically intact stimulating cells (13, 14). Therefore, the failure of B cells to participate in the proliferation in MLC cannot be attributed to unrecognized B-cell injury; rather, it expresses the true property of B cells, i.e., their nonreactivity to alloantigens in MLC. Our results in man are in accordance with most studies in experimental animals, reporting T-cell proliferation in MLC (15–17); reports that both T and B cells proliferate in MLC (18–20) are controversial (15).

Based on these findings and previous reports, the cellular cooperative events ultimately leading to proliferation in MLCs can be outlined as follows: Stimulating alloantigens are predominantly located on B cells (references 21 and 22, and footnote 2), but only T cells possess the capacity to recognize (23) these alloantigens, and to respond to them by proliferation. However, the presence of monocytes is required for this T/B-cell interaction (references 24–28, and footnote 3), although their precise role remains to be identified.

SUMMARY

The nature of the cells which proliferate in response to allogeneic histoincompatible lymphocytes in human mixed leukocyte cultures (MLC) was investigated. Direct information on the differentiation of proliferating cells was obtained in an in vitro chimeric system with T- and B-cell sex chromosome markers. Highly purified T and B cells were prepared from the peripheral blood of three pairs of HL-A genotype identical, MLC-negative siblings of opposite sex by E-rosette sedimentation techniques. Recombined T and B cells of the siblings were stimulated with allogeneic cells, and karyotype analysis of pro-

liferating cells performed. 96% of the proliferating cells were found to carry the sex chromosome marker of the respective T-cell population. These results indicate that proliferation in human MLCs is a feature of T cells.

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