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Synergism of Stimulating Capacity of Pooled Lymphocytes in the Mixed Lymphocyte Reaction

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Summary. One-way mixed lymphocyte reactions in cultures of responding cells from a single subject and a pool of stimulating cells from two or more donors exhibited a synergistic response. Increased activity of the responding cells showed a positive correlation with the pool size. Treatment of the responding cells, stimulating cells or both with neuraminidase significantly increased the response.

INTRODUCTION

The mixed lymphocyte reaction (MLR), described by Bain, Vas and Lowenstein (1964), has shown considerable promise as a means of pairing potential transplant donors and recipients (Bach, 1970; Bach, Debray-Sacchs, Crosnier, Kreis and Dormont, 1970). The MLR is recognized as being primarily dependent upon cell-bound antigens determined by the histocompatibility locus A (HL-A) and the magnitude of this reaction shows a positive correlation with the degree of HL-A disparity of the two subjects (Schellekens, Vriesendorp, Eijsvoogel, Van Leeuwen, Van Rood, Miggiano and Cepellini, 1970).

The sensitivity of the MLR as a histocompatibility assay is demonstrated by the fact that cultures of lymphocytes from HL-A identical individuals rarely show any reaction; however, significant lymphocyte activation is observed in MLR of subjects exhibiting a disparity of only one HL-A allele (Park and Good, 1972).

Since more than thirty antigens of the human leucocyte have been identified that belong to the HL-A system (Histocompatibility Workshop, 1970), it would seem reasonable that significantly higher MLR would be observed in cultures containing a pool of lymphocytes from a number of individuals. Accordingly, we conducted experiments to quantitatively evaluate lymphocyte reactivity to a test battery consisting of pooled cell suspensions from a number of subjects. Presented here are the results of these studies demonstrating the synergism of the stimulating capacity of such a pool.

MATERIALS AND METHODS

Mixed lymphocyte cultures were prepared using a simplified whole blood culture method developed in our laboratory (Pauly and Sokal, 1972). Heparinized blood (10 u/ml), collected from randomly selected healthy donors, was diluted 1:10 with unsupplemented medium RPMI 1640 containing 100 u of penicillin and 50 μg of streptomycin per millilitre. One-way MLR were prepared in 16 × 125-mm polystyrene culture tubes by adding 1·5 ml of a cell suspension from a single individual (responding cells) and 1·5 ml of an

irradiated (6000 r) unpooled or pooled cell suspension from one or more individuals (stimulating cells). Corresponding 3·0 ml control cultures of an irradiated or non-irradiated cell suspension were prepared so as to contain numbers of lymphocytes equal to those present in the mixed cell cultures. The loosely capped cultures, established in triplicate, were placed in an incubator at 37° with a continuous flow of humidified 5 per cent CO_2 in air. One microcurie of ^{3}H-thymidine, with a specific activity of 2·0 Ci/mM, was added to each of the cultures 24 hours prior to harvesting after 7 days of incubation. At harvest, the cells were transferred to glass conical centrifuge tubes and washed twice with cold 3 per cent acetic acid by centrifugation at 350 *g* for 8 minutes. This resulted in lysing the majority of erythrocytes, leaving the leucocytes intact and free of non-incorporated isotope. The resulting cell button was bleached with 0·05 ml of 30 per cent hydrogen peroxide and heated in a drying oven at 85° for 20 minutes. The cells were then dissolved with 0·6 ml of NCS solubilizer (Amersham/Searle Corporation, Arlington Heights, Illinois) and incorporated into 10 ml of a 24-fold dilution of Spectrafluor (Amersham/Searle). Radioactivity was measured in a Nuclear-Chicago Mark I liquid scintillation counter and the results expressed as counts per minute (cpm). The blastogenic index was defined as the ratio of radioactivities in mixed cell cultures minus irradiated cultures to radioactivities in non-irradiated control cultures.

RESULTS

Lymphocyte activation responses of twelve subjects to both phytohaemagglutinin (PHA) and a pool of irradiated cells prepared from these same donors is shown in Table 1. The mean ^{3}H-thymidine incorporation for PHA-activated cultures was $91·5 \times 10^3$ cpm or approximately 130 times greater than that recorded in the corresponding unstimulated controls. A high degree of activity was also observed in all of the one-way MLR which showed a mean response approximately 42 times greater than that of the controls. The strength of the MLR is further illustrated by the fact that individual values were from 20·3 to 56·9 per cent of the corresponding PHA responses. Results of corresponding

TABLE 1

PHA-INDUCED BLASTOGENESIS AND ONE-WAY MLR AGAINST POOLED LYMPHOCYTES

Responding cell donor	Thymidine incorporation, 10^3 cpm			
	None	PHA	Pooled lymphocytes*	
J.P.	0·6	71·7	17·0	(23·7)
M.G.	0·3	87·8	27·9	(31·8)
B.H.	1·2	100·2	36·7	(36·6)
B.D.	0·4	63·9	33·5	(52·4)
C.W.	0·5	59·5	28·1	(47·2)
D.B.	1·4	59·0	33·6	(56·9)
T.H.	1·1	130·2	41·8	(32·1)
J.S.	0·7	120·5	37·0	(30·7)
J.W.	1·5	138·5	30·4	(20·3)
J.I.	0·3	87·0	28·5	(32·8)
L.M.	0·4	104·4	21·7	(20·8)
M.V.	0·2	75·7	19·7	(26·1)
Mean	0·7	91·5	29·7	(34·3)

* Irradiated suspension from the above twelve donors.
† Per cent of PHA response.

stimulated and unstimulated cultures containing plasma or erythrocytes from the pooled bloods showed no change in the ^{3}H-counts. Furthermore, the MLR was not affected by either ABO or Rh blood group incompatibilities.

Results of comparative one-way MLR experiments of three subjects (B.B., T.C. and A.K.) to single, double or triple stimulating cell types showed a significant synergistic effect of the stimulating capacity of the pooled cells (Table 2). For example, subject B.B.

TABLE 2

COMPARISON OF ONE-WAY MIXED LYMPHOCYTE REACTION BETWEEN SINGLE RESPONDING CELL TYPE AND SINGLE OR MULTIPLE STIMULATING CELL TYPES

Stimulating cell donor (irradiated cells)	Blastogenic index[a] (per cent enhancement)[b] Responding cell donor; 1·50 ml					
	B.B.		T.C.		A.K.	
*J.P.	28·7		41·6		7·0	
*T.H.	28·4		7·6		35·7	
*M.G.	15·0		84·3		38·7	
†J.P.+T.H.	54·2	(190)	58·8	(239)	30·1	(141)
†J.P.+M.G.	84·3	(386)	85·1	(135)	22·1	(97)
†T.H.+M.G.	125·5	(578)	69·7	(152)	42·5	(114)
‡J.P.+T.H.+M.G.	140·2	(583)	68·0	(153)	39·2	(144)

[a] Blastogenic index (BI) $= \frac{\text{cpm mixed cell culture} - \text{cpm irradiated cell culture}}{\text{cpm of responding cell cultures}}$

[b] Per cent enhancement $= \frac{\text{BI of multiple stimulating cell types}}{\text{BI of mean individual stimulating cell types} \times 100}$

* = 1·50 ml cell suspension.
† = 0·75 ml cell suspension from each of two donors.
‡ = 0·50 ml cell suspension from each of three donors.

showed blastogenic indicies of 28·7 and 28·4 to irradiated cell suspension from J.P. and T.H., respectively. However, the MLR of B.B. with a pooled cell suspension from both J.P. and T.H. was 54·2; 190 per cent higher than the predicted blastogenic response of approximately 28·6. Similarly, a blastogenic index of 140·2 was observed using a pool of cells from three donors (J.P., T.H. and M.G.). If the lymphocyte response of B.B. to this cell pool was additive, an index of 72·1 (the sum of 28·7, 28·4 and 15·0) would have been anticipated. Comparison of the index value of 140 with the mean blastogenic index of the individual cell types showed a 583 per cent enhancement of lymphocyte activation.

The results of similar one-way MLR of five subjects to a pool from 1 to 8 donors are presented in Table 3. In each of these cases, a positive correlation was observed between

TABLE 3

ONE-WAY MLR BETWEEN SINGLE RESPONDING CELL TYPE AND VARIOUS NUMBERS OF STIMULATING CELL TYPES*

Responding cell donor*	Blastogenic index Number of stimulating cell donors*			
	1	2	4	8
A.K.	2·1	2·5	15·8	53·1
B.P.	1·6	6·1	23·0	59·5
T.R.	8·8	30·7	49·9	86·2
M.M.	11·0	13·1	64·1	137·7
E.C.	19·7	31·4	59·1	149·3
Mean	8·6	16·7	42·4	97·2

*Responding and stimulating cell donors were unrelated.

TABLE 4

ENHANCEMENT OF LYMPHOCYTE RESPONSE AGAINST POOLED LYMPHOCYTES BY *Vibrio cholerae* NEURAMINIDASE

Composition of culture*	^{3}H-thymidine uptake ($\times 10_3$ cpm)	Blastogenic index
RC (PBS)	190	—
RC (VCN)	160	—
PSC (PBS)†	133	—
PSC (VCN)†	146	—
RC (PBS) + PSC (PBS)†	9,059	47·0
RC (PBS) + PSC (VCN)†	14,095	73·4
RC (VCN) + PSC (PBS)†	15,654	97·0
RC (VCN) + PSC (VCN)	13,842	85·6

* Two 3-ml aliquots of heparinized blood was centrifuged at 200 *g* for 10 minutes and the plasma removed. One cell pellet was then incubated with 4 u of neuraminidase (VCN) per ml of blood for 30 minutes at 37°. The corresponding control cell pellet was treated in the same manner with phosphate buffered saline (PBS). Both cell pellets were then washed twice with 12 ml of PBS and the resulting cells reconstituted with an equal volume of plasma collected prior to treatment. Whole blood cultures were then prepared in the usual manner.

† Cell suspension irradiated with 6000 r.

RC = responding cells.

PSC = pool of stimulating cells from three unrelated donors.

the magnitude of the MLR and the number of stimulating cell donors. The blastogenic indicies of these individuals to a cell suspension from eight subjects were 53·1, 59·9, 86·2, 137·7 and 149·3. The mean blastogenic index of 96·8 is considerably higher than that usually observed for lymphocyte activation responses induced with soluble antigens such as tuberculin-PPD, mumps, etc.

Additional experiments were conducted to determine whether or not treatment of the cells with *Vibrio cholerae* neuraminidase (VCN) would affect the one-way MLR. The results of a representative study using four different combinations of single responding cell type and pooled stimulating cell types, treated with either VCN or phosphate buffered saline (PBS), are presented in Table 4. In all instances, treatment of the stimulating cells, responding cells or both types with VCN significantly enhanced the one-way MLR; the recorded values were 153–173 per cent higher than the MLR of the corresponding PBS-treated cultures. No appreciable change was observed in unstimulated cultures of cells treated with VCN or PBS.

DISCUSSION

The results of this study demonstrate the synergistic effect of the stimulating capacity of pooled lymphocytes in the one-way MLR. Furthermore, the intensity of the MLR showed a positive correlation with the size of the pool. These responses could not be attributed to either the plasma or red blood cells of the pooled blood. ABO and Rh blood group incompatibilities were also observed to have no significant influence on these reactions. The kinetics of the MLR, i.e. optimal cell density and day of maximal response, were not altered by changing the size of the stimulating cell pool. Since the number of stimulating and responding cells present in all experiments were held constant, the responses observed in the MLR undoubtedly represent a manifestation of lymphocyte activation by cell-bound histoincompatible antigens.

Of interest is the observation that when a relatively large pool size was employed, the magnitude of the one-way MLR was significantly greater than that usually observed with soluble antigens and in some instances approximated the magnitude of response often observed with potent nonspecific stimulants such as pokeweed mitogen, staphylococcal filtrate and Concanavalin A.

Treatment of the cells in the one-way MLR with VCN significantly enhanced lymphocyte activation. This enhanced reactivity is in good agreement with results of previous studies and is probably related to the expression of additional cell-bound antigens or other physical and functional changes in the membrane following the removal of sialic acid by this enzyme (Han, 1972; Lundgren and Simmons, 1971).

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