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POTENTIATION OF LYMPHOCYTE

TRANSFORMATION BY MEMBRANE-MEMBRANE INTERACTION

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MASTER

In the search for a better understanding of the mitogenic activity of (PHA) phytohemagglutinin/derived from Phaseolus vulgaris, our laboratory has developed a method for the purification of the mitogenic proteins present in PHAP, a commonly employed commercial preparation obtained from Difco Laboratories. By a combination of CM Sephadex chromatography, followed by molecular sieving over Sephadex G-150, we have isolated two distinct mitogenic proteins from such preparations. (1) Figure 1 illustrates the results obtained from the final step of the purification procedure, and shows an analysis of the purified mitogens obtained when they are subjected to acrylamide gel electrophoresis, comparing them with the starting material. These mitogenic proteins have been designated high titer hemagglutinating PHAP (or H-PHAP) and low titer hemagglutinating PHAP (or L-PHAP). Table 1 summarizes the serologic and mitogenic properties of these two proteins.

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Table 1

SEROLOGIC AND MITOGENIC POTENCY OF L- AND H-PHAP

	L-PHAP	H-PHAP
1. Hemagglutination Unit μg/ml	227	1.05
2. Leukoagglutination Unit μg/ml		
a. Mixed leukocytes	5.6	69.5
b. Lymphocytes	11.3	304
3. Mixed Agglutination Unit (Lymphocyte-Erythrocyte) μg/ml	40	0.3
4. Transformation Unit μg/ml	0.3	1.05

Both are potent mitogens and are also capable of leuko- and lympho-agglutination. H-PHAP, however, is a much more potent red cell agglutinin than L-PHAP, and is also capable of producing mixed red cell-lymphocyte agglutination at very high dilutions. L-PHAP has a molecular weight of 115,000 and contains 4% carbohydrate by weight. H-PHAP is a somewhat larger molecule as determined by molecular sieving and contains approximately twice as much carbohydrate.

Both L- and H-PHAP were labeled with [^{125}I]. Figure 2 shows that over 90% of H-PHAP can be adsorbed by red blood cells while only 3-4% of L-PHAP can be adsorbed in the same fashion. Table 2 reveals that the mitogenic capacity of H-PHAP is almost totally removed by prior red cell adsorption, while the mitogenic capacity of L-PHAP is unaffected by prior exposure to red cells.

Table 2

ADSORPTION OF MITOGENIC ACTIVITY BY RED BLOOD CELLS

PHA Type	Packed RBC Added (ml)	[¹⁴ C]thymidine Incorporation (CPM)	% Inhibition
† L-PHAP	-	23,925	-
	0.05	23,643	1
	0.1	23,377	2.3
* H-PHAP	-	38,396	-
	0.05	2,163	95.7
	0.1	1,182	98.3

Following adsorption of mitogen with the indicated volume of RBC for 30' at 25°, the red cells were removed by centrifugation and the supernatant was added to 2×10^6 lymphocytes in 4 ml MEM containing 12-1/2% autologous plasma. [¹⁴C]thymidine incorporation was determined as outlined in Reference 1.

† 21 µg

* 17 µg

During our early studies on the comparative mitogenic potency of both H- and L-PHAP we noted that although L-PHAP was a more potent mitogen in the lower regions of the dose response curve, the plateau value of peak response was always higher with H-PHAP. In view of the marked interaction of H-PHAP with red blood cells and its potency in mixed lymphocyte-erythrocyte agglutination, and since our early experiments were done with lymphocytes that were contaminated with 3-5 times their number of erythrocytes, we decided to investigate the effects of the presence of red blood cells on lymphocyte transformation. Pure lymphocytes, entirely free of red cells or red cell stroma, and containing only 20-40 platelets/100 lymphocytes, were

obtained by means of gravity sedimentation of whole blood, nylon column passage, osmotic lysis of red blood cells, and centrifugation through a saline-plasma gradient. (1) We used these lymphocytes to study the ability of autologous red blood cells to influence human lymphocyte transformation by both the L- and H-PHAP mitogens, and found that although red blood cells had no effect on lymphocyte transformation by L-PHAP, they markedly potentiated the effects of H-PHAP.

Figure 3 illustrates the results of an experiment with varying doses of mitogen upon the transformation of 2 million lymphocytes with and without the addition of 20 million erythrocytes. The presence of red cells does not substantially affect the transformation of lymphocytes when L-PHAP is the mitogen. The ability of H-PHAP to stimulate DNA synthesis in lymphocytes, however, is markedly enhanced by the presence of red cells. This effect is most marked at low and intermediate points on the dose response curve and diminishes as the plateau of stimulation is approached. Figure 4 illustrates the effect of varying numbers of red blood cells on the transformation of 2 million lymphocytes in the presence of a constant amount of mitogen. The dose of mitogen chosen was calculated to give from $1/3$ to $1/4$ of the peak response obtainable in the absence of red blood cells. Here again the presence of red blood cells did not alter the ability of L-PHAP to transform lymphocytes, although a ratio of red cells to lymphocytes as low as 1:1 increased lymphocyte transformation by almost 300% when H-PHAP was employed. The plateau of stimulation by red cells was approached at a red cell:lymphocyte ratio of 10:1. In other experiments the presence of as few as 1 red cell for every 10 lymphocytes resulted in a 2-fold increase of lymphocyte transformation (Table 3).

Table 3

ENHANCEMENT OF LYMPHOCYTE TRANSFORMATION BY RED CELLS

A. Constant Red Cells (10×10^6); Varying H-PHAP Dose

PHA Dose μg	CPM [*] Red Cells Present	CPM [*] Red Cells Absent	Stimulation Index
2.7	6,166	416	1482
5.4	32,373	4,974	651
13.4	38,909	17,003	229
26.8	39,377	25,377	155
40.2	39,899	29,858	133.6

B. Constant H-PHAP Dose (5.4 μg); Varying Red Cells

Red Cells (millions)	CPM [*]	Stimulation Index [†]
0	4,974	-
10.0	32,373	651
5.0	22,618	455
2.0	18,917	380
1.0	13,989	281
0.5	14,817	298
0.2	10,681	215

Human lymphocytes (2×10^6) were cultured with and without red cells.

^{*}DNA synthesis as measured by [^{14}C]thymidine incorporation.

[†] $\frac{\text{CPM Red Cells Present}}{\text{CPM Red Cells Absent}} \times 100.$

As shown in Table 4, the ability of red cells to potentiate the transformation of lymphocytes by H-PHAP was limited to red cell stromal particles derived from osmotic lysis of red cells; stroma free hemolysate displayed no potentiating capacity. It is noteworthy that the potentiation efficiency of

Table 4

ENHANCEMENT OF LYMPHOCYTE TRANSFORMATION BY RED CELLS AND RED CELL STROMA

	RBC or RBC Equivalent Added	Number of Red Cells Added (millions)	H-PHAP Dose μg	[¹⁴ C]thymidine Incorporation CPM	Stimulation Index
A. RBC		20	-	320	-
B. None		-	5.4	9,447	-
C. RBC		20	5.4	52,284	553.4
RBC		2	5.4	33,387	353.4
D. RBC Stroma		84	5.4	33,465	354.2
RBC Stroma		42	5.4	26,578	281.3
RBC Stroma		21	5.4	18,712	198.1
RBC Stroma		10.5	5.4	13,688	144.9
RBC Stroma		5.25	5.4	10,039	106.3
RBC Stroma		2.6	5.4	8,633	91.4
E. RBC Hemolysate		74	5.4	8,376	88.7
RBC Hemolysate		37	5.4	7,421	78.6
RBC Hemolysate		18.5	5.4	6,988	74

Hemoglobin-poor stroma was prepared by osmotic lysis of red cells in dilute phosphate buffer. The stromal particles were harvested and washed 3X by ultracentrifugation prior to use. The supernatant from the first centrifugation was used as hemolysate. Stromal particles were enumerated in a Coulter Counter. Two million lymphocytes were employed in each culture.

red cell stromal particles was only 1/40 that of intact red cells, since 84 million stromal particles were required to achieve the same potentiation as

two million intact red cells. Studies with [^{32}DFP] labeled red cell stroma, however, disclosed that a maximum of 1/12 of the added stromal particles became attached to or sedimented with lymphocytes; the remainder were found free in the supernatant medium (Table 5). Other studies with [^{51}Cr] labeled red blood

Table 5

CORRELATION BETWEEN REC STROMA ENHANCEMENT OF LYMPHOCYTE TRANSFORMATION
AND SEDIMENTABLE [^{32}DFP] STROMA

PHAP Type	Lymphs (2×10^6)	Stromata (264×10^6)	[^{14}C]thymidine Incorporation	Stimulation Index	Sedimentable Stromal Particles	
					%	No. ($\times 10^6$)
None	+	+	-	-	0.6	
	-	+	-	-	0.4	
H-PHAP (4 μl)	+	-	16,086	-	-	
	+	+	41,960	261	8.3	21.9
	-	+	-	-	7.3	19.3
L-PHAP (2 μl)	+	-	13,302	-	-	
	+	+	12,965	97.5	0.4	
L-PHAP (20 μl)	-	+	-	-	0.5	

Red cells were labeled in vitro with [^{32}DFP] and stromal particles were prepared and added to replicate cultures with and without PHAP and/or lymphocytes. At the conclusion of the experiment, the cultures were centrifuged at 300 X g for 10 minutes and the supernatants were discarded. The cell buttons were washed 3X in saline, and then assayed for [^{32}P] and [^{14}C] in a liquid scintillation counter.

cells have shown that the potentiation of lymphocyte transformation by H-PHAP is associated with erythrocyte injury as measured by [^{51}Cr] release; red cell injury does not occur if lymphocyte transformation in the presence of red cells is effected by L-PHAP (Table 6).

Table 6

CORRELATION BETWEEN RBC POTENTIATION OF LYMPHOCYTE TRANSFORMATION
AND RBC INJURY ($[^{51}\text{Cr}]$ RELEASE)

PHAP Type	PHAP μl	$[^{14}\text{C}]$ thymidine Incorporation (CPM)		Stimulation Index	$[^{51}\text{Cr}]$ Release (%)	
		Lymphs	Lymphs + RBC		RBC	RBC + Lymphs
None	-	-	-		7.3	7.4
H-PHAP	4	8,686	57,259	659	8.0	14.1
	20	43,723	54,608	125	7.7	18.0
L-PHAP	2	5,174	5,130	99	7.4	7.0
	10	31,713	28,922	91	7.3	7.1

After the period of culture, the supernatant fluid was removed and assayed for $[^{51}\text{Cr}]$ content in a NaI crystal gamma counter.

These observations on the potentiation of lymphocyte transformation by red cells solely in the presence of that mitogen capable of bringing red cells and lymphocytes into closely fixed apposition suggested to us that membrane-membrane interaction between lymphocytes and red blood cells, as well as between lymphocytes and lymphocytes, played an important role in the stimulation of DNA synthesis and transformation by mitogen. We reasoned that if this were a general feature of lymphocyte transformation by mitogen, then it should be possible to demonstrate a similar phenomenon with the L-PHAP mitogen, providing that a suitable membrane-bearing particle were used as stimulant and providing also that such a particle, like the red cell, was incapable of responding to L-PHAP by DNA synthesis. The particle chosen for this experiment was the ^{autologous} x-irradiated lymphocyte. Figure 5 shows the effect of x-irradiation of lymphocytes on the subsequent capacity of these cells to respond to mitogen as measured by DNA synthesis. This curve, which represents the average of 3

separate experiments, determined the use of a dose of 5000 R in order to reduce DNA synthesis to less than 3% of the control value in the "nonresponding" lymphocyte particles. Table 7 summarizes the results obtained when 1 million normal lymphocytes were exposed to varying doses of both L- and H-PHAP mitogens in the absence of, and in the presence of, an equal number of nonresponding autologous irradiated lymphocytes. The results clearly demonstrate that the presence of these inert lymphocytes markedly potentiated the mitogenic capacity of both L- and H-PHAP. This potentiation resembled that obtained

Table 7

POTENTIATION OF LYMPHOCYTE TRANSFORMATION BY X-RAY KILLED LYMPHOCYTES

[¹⁴ C]thymidine Incorporation(CPM)					
PHAP Type	PHAP μl	Normal Lymphs	Normal Lymphs + X-Ray Lymphs (Theoretical) [†]	Normal Lymphs + X-Ray Lymphs (Observed)	Stimulation ^Δ Index
H-PHAP [*]					
	4	6,041	6,597	25,036	380
	10	19,556	20,509	29,612	144
	40	31,464	32,417	34,617	107
L-PHAP [†]					
	1	1,094	1,835	8,062	439
	2	3,734	4,475	17,817	398
	10	21,620	22,514	24,166	107

Lymphocytes (1×10^6) were cultured with and without the addition of an equal number of autologous lymphocytes that had been exposed to a dose of 5000 R.

* 1.34 mg/ml

† 1.54 mg/ml

† This figure accounts for the small amount of residual DNA synthesis observed when X-irradiated lymphocytes were cultured separately.

$$^{\Delta} \text{Stimulation Index} = \frac{\text{CPM observed}}{\text{CPM theoretical}} \times 100$$

with red cells in the presence of H-PHAP in that it was most marked at low and intermediate points on the dose response curve and diminished as the plateau peak response was approached.

SUMMARY

In summary, we have shown that membrane-membrane interaction brought about by phytohemagglutinin molecules acting as intercellular ligands is an important mechanism for the potentiation of lymphocyte transformation, and that such potentiation occurs even with the cooperation of a mitotically inert particle lacking genetic information such as the red cell or red cell stromal particle. In all probability the increase in DNA synthesis results not from increasing the extent to which any single cell synthesizes DNA, which remains an all or none phenomenon, but rather through an increase in the efficiency with which individual cells are recruited into the transformation process by a mechanism involving cell-cell, or more specifically, membrane-membrane interaction. These observations may have some bearing on the experiments of Fisher and Mueller which indicate that the lymphocyte membrane undergoes significant modulation of membrane phospholipids as one of the earliest steps in the process of transformation by phytohemagglutinin. (2) They also fit nicely into the growing body of evidence which indicates that the initiation and perpetuation of the immune response by lymphocytes requires the interaction of two or three varieties of macrophage and/or lymphocytes brought into close and specific contact by antigen and/or antibody molecules acting as ligand. (3,4,5,6,7) In addition to the possibility of information transfer, such cell-cell interaction may act to provoke the derepression of the lymphocyte, and thus enable it to synthesize macromolecules and divide. When the ligands and cell receptors are specific, as in the immune response, the result is specific, namely antibody synthesis.

When, however, the ligand and receptor are immunologically nonspecific, as in the case of phytohemagglutinin-induced transformation, the result is abortive.

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Legends for Figures

Figure 1

Sephadex G-150 chromatography of L-PHAP and H-PHAP pools derived from CM Sephadex fractionation of PHAP. From 2 - 40 μ l aliquots were used in determining the specific activity of transformation for L-PHAP fractions across the elution peak. The L-PHAP and H-PHAP preparations were pooled and concentrated, and compared with the starting material by acrylamide gel electrophoresis (shown on right). The Sephadex G-150 elution peaks of human gamma globulin (γ G) and bovine serum albumin (BSA) are also shown.

Figure 2

Red cells were incubated with either [125 I] H- or L-PHAP in the amounts shown at 25° for 60 minutes. Following centrifugation and washing 3X, the red cell buttons were lysed with 2 ml of 1% Triton X-100 and counted in a NaI crystal gamma detector. Both L- and H-PHAP retained full mitogenic capacity after radioiodination.

Figure 3

The potentiation of lymphocyte transformation by red cells. Lymphocytes (2×10^6) were transformed by varying doses of L- or H-PHAP in the presence or absence of 20×10^6 erythrocytes. One-tenth microcurie [14 C]thymidine was added to each culture at 72 hours, and the cells were harvested for liquid scintillation counting 16 hours later. See Reference 1 for details of the procedure. Stimulation Index = $\frac{\text{CPM} + \text{RBC}}{\text{CPM} - \text{RBC}} \times 100$.

Figure Legends--(continued)

Figure 4

The potentiation of lymphocyte transformation by red blood cells. In this experiment the dose of H- or L-PHAP was kept constant and varying numbers of red cells were added to 2×10^6 lymphocytes.

Figure 5

The effect of in vitro irradiation of human lymphocytes on their susceptibility to transformation by phytohemagglutinin.

RBC UPTAKE OF $[^{125}\text{I}]$ H-AND L-PHAP

2.0
1.5
1.0
0.5
0
ug/unit