A Quantitative Human Mixed Lymphocyte Culture Using Suspension Cultures[1] (37658)

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(Introduced by R. P. Geyer)
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Several investigators have concentrated on studying the genetic factors which determine the mixed lymphocytic culture (MLC) response and have shown a significant correlation between the magnitude of the MLC response and the HL-A antigens. Nonstimulation was observed between lymphocytes of HL-A identical siblings while the MLC response was stronger between siblings differing by two than by one HL-A allele (1, 2). Subsequently it was found that the genetic loci determining the HL-A antigens and MLC response are separate but closely linked (3). The latter observation could explain the uncommon occurrence of an MLC response in HL-A identical mixed lymphocytes. The above observations suggest that it is possible to design and use the MLC to determine differences of HL-A incompatibility quantitatively. In a previous report (4), factors other than genetic ones were found to influence markedly the magnitude of the MLC response during a 7-day incubation period, *i.e.*, the pH of the medium, the number of responding and stimulating cells, changes of the medium, mitomycin C treatment, and the method of purifying lymphocytes (4). It was also found that maximum discrimination between MLC responses of various magnitude occurred on the 7th day of incubation. The latter investigations led to a method of human MLC which will permit the *quantitative* determination of histoincompatibility differences between related and unrelated persons. In the present report a new method of MLC using suspension cultures of lymphocytes is described. The latter increased the magnitude of the MLC response as determined on the 7th day of incubation on the average six times, as compared to cultures with settled cells and provides a more sensitive means to discriminate *quantitatively* between histoincompatibility differences.

Materials and Methods. The method to purify lymphocytes from peripheral blood, the preparation of stimulating lymphocytes, the conditions of cultivation, and the determination of DNA have been described (4). The buffer Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma Chemical Co., St. Louis, Missouri) was added to the culture medium in a concentration of 25 m*M*. Two types of culture conditions were used. In the first condition cells were incubated in 16 × 125 mm round bottom stoppered glass tubes containing 3 ml of medium. In the latter tubes the lymphocytes settled on the bottom of the tubes during incubation. In the second condition the lymphocytes were incubated in 11 × 77 mm closed polypropylene tubes (Arthur H. Thomas Co., Philadelphia, PA, Cat. No. 2602-B 16 and 2602-C 20) of 3.5 ml content and filled with 3 ml of medium. The polypropylene tubes were incubated standing for 5 days and were then rolled on a tissue culture roller drum rotating at a speed of 0.2 rpm from days 5–7. Continuous rotation of tubes, completely filled with medium, keeps the cells continuously suspended in the medium. All cultures were incubated for 7 days and were labeled during the 7th day for 24 hr with ^{3}H-thymidine (SA 6.7 Ci/mmole, New England Nuclear Corp., Boston, MA). All of the

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TABLE I. Comparison of the Magnitude of the MLC Response in Cultures with Settled and Suspended Lymphocytes.[a]

Tube	Combination number 1	2	3	4	5	6
	MLC response: cpm/culture × 10^3					
A	4.5 (.2)	8.3 (.5)	7.1 (.5)	1.8 (.2)	4.7 (.7)	7.2 (.6)
B	6.0 (.2)	49.0 (.5)	17.5 (.6)	6.5 (.2)	22.1 (.6)	20.3 (.4)
	Combination number 7	8	9	10	11	12
	MLC response: cpm/culture × 10^3					
A	10.1 (.7)	7.3 (.5)	12.4 (1.0)	3.7 (.4)	6.2 (.5)	10.0 (1.0)
B	11.0 (.7)	19.7 (.6)	33.3 (1.1)	36.0 (.3)	10.0 (.6)	47.6 (1.0)

[a] Twelve different allogeneic combinations of 1×10^5 R and 5×10^5 S were incubated in settled (A) and suspended (B) cell cultures. Parallel control tubes containing 1×10^6 R and 5×10^5 S autologous lymphocytes were included (data in parentheses). The data given are the responses in allogeneic cultures from which the responses in control cultures have been subtracted.

data in the figures and tables are averages of three replicate cultures. The variation of counts among the replicate cultures rarely exceeded 15%.

Results. Influence of settled vs suspended lymphocytes. The MLC response of allogeneic mixtures of 1×10^5 responding (R) and 5×10^5 stimulating (S) lymphocytes was compared in cultures containing settled cells with cultures containing the lymphocytes suspended in the medium (see Materials and Methods). In 12 allogeneic combinations of lymphocytes it was found (Table I) that the MLC response was on the average three times greater in suspension cultures as compared to settled cell cultures. No significant difference was found between settled and suspended cell cultures of autologous controls containing 1×10^5 R and 5×10^5 autologous S lymphocytes.

Influence of the number of S lymphocytes. The results of Fig. 1 showed that increasing numbers of S cells produced a stronger response in suspension cultures than in cultures with settled cells. Indeed, increasing the number of S cells from 3 and 6×10^5 to 9 and 12×10^5 S lymphocytes stimulated the response of suspended cells further, while a similar increase of S cells increased only slightly or not at all the response of settled cells. The response caused by 9 and 12×10^5 S lymphocytes in suspension cultures was more than twice the response in parallel settled cell cultures. The results shown in Fig. 1 are representative of 6 allogeneic combinations. In all of the combinations studied, suspension increased only the response in allogeneic mixtures with no significant change in the response of the controls, containing similar numbers of autologous R and S lymphocytes. Thus the combined use of suspending the cell mixtures (see above) and increasing the number of stimulating cells increased the MLC response approximately 6 times as compared to settled cell mixtures.

Influence of the pH of the medium. The determination of the maximum MLC response in suspension cultures in closed tubes was possible only when the pH of the medium had been stabilized with the buffer Hepes. During a 7 day incubation period the pH of the medium without Hepes in capped cell cultures placed in a CO_2 atmosphere shifted to the acid side, and a maximum response was obtained only when the pH was maintained between 7.20–7.45; outside of this pH range the MLC response was markedly reduced (Fig. 2, lower two curves and Ref. 3).

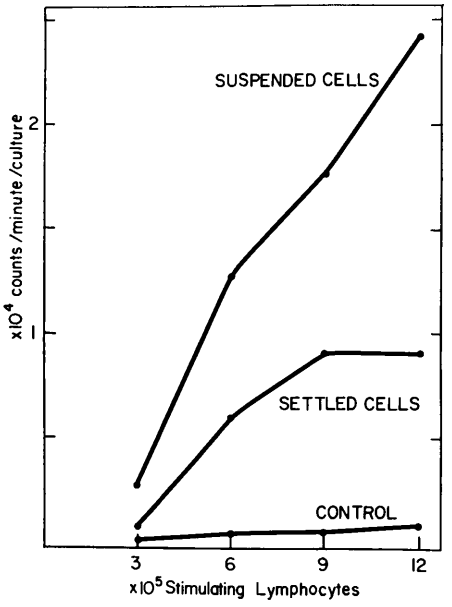

FIG. 1. Influence of the number of S lymphocytes in settled and suspended cell cultures. Inocula of 1 × 10^5 R and increasing numbers of allogeneic S lymphocytes were incubated in parallel settled and suspended cell cultures. Parallel control tubes containing 1 × 10^5 R and similarly increasing numbers of autologous S cells were incubated. The control data for settled and suspended cell cultures were similar and are represented by the lower curve.

In closed tubes the pH could not be maintained within the above limits and shifted to below 7.20, unless Hepes had been added. A concentration of 25 m*M* Hepes stabilized the pH to within 0.1–0.2 of a unit of pH throughout a 7-day incubation period. When the replicate mixtures of allogeneic lymphocytes were incubated in medium containing 25 m*M* Hepes at pH values ranging from 7.00–7.90, two patterns of MLC responses occurred among a total of 19 combinations examined (Fig. 2, upper 2 curves); in one series of combinations, the maximum response occurred approximately between pH values of 7.20–7.90, in the second series the maximum response occurred only between a pH of 7.50 and 7.70 to 7.80. Both patterns occurred in settled and suspended cell cultures. The reason why certain mixtures of allogeneic lymphocytes responded differently than others to the pH of the medium was not clear.

Influence of the concentration of plasma in the medium. Replicate suspension cultures containing 1 × 10^5 R and 10 × 10^5 S lymphocytes were incubated in medium containing 10, 20, 30, 40, 50, and 60 ml of fresh human plasma per 100 ml of complete medium. The results (Fig. 3) showed that increasing the concentrations of plasma increased

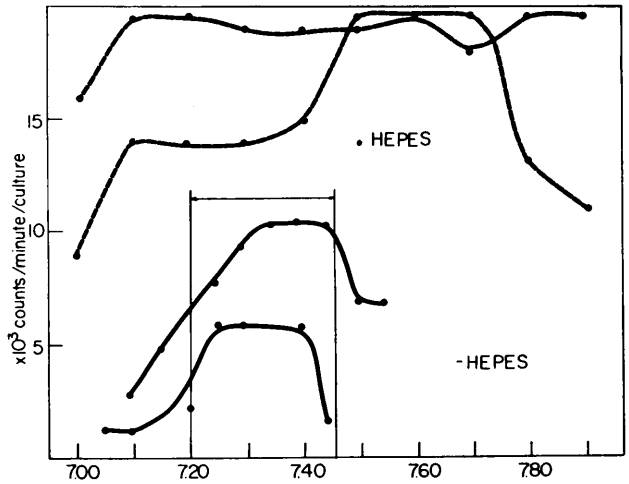

FIG. 2. Upper 2 curves: two allogeneic combinations of 2 × 10^5 R and 6 × 10^5 S lymphocytes were inoculated in parallel suspension cultures containing media of increasing pH values, shown in the abscissa. 25 m*M* Hepes was added to the medium. The lower curves represent 2 similar but separate experiments of allogeneic settled lymphocytes incubated in medium without Hepes. The latter experiments have been described in a previous report (4) and are included here for comparison.

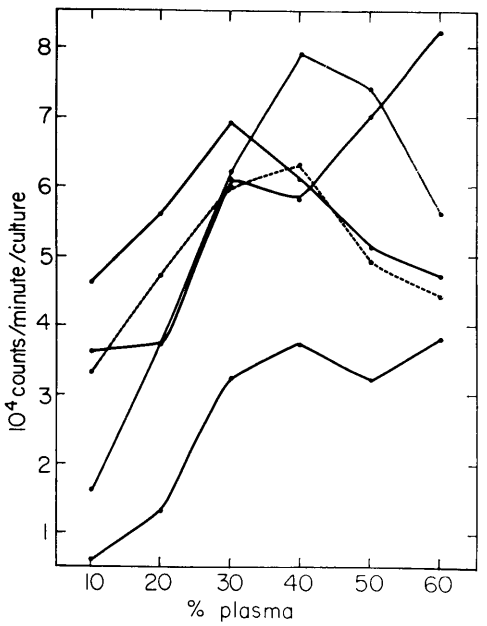

FIG. 3. Influence of the concentration of plasma in the culture medium. Five different allogeneic combinations of 1 × 10^5 R and 9 × 10^5 S lymphocytes were incubated in increasing concentrations of plasma.

the MLC response until a maximum was reached at a 30–40% concentration; higher concentrations did not stimulate the response further except in one mixture. Additional experiments, using 2, 4, 6, and 8 μCi of ^{3}H-thymidine on day 7, showed that the amount of ^{3}H-thymidine incorporated into DNA was of similar magnitude in all cultures, except those containing 2 μCi, where the incorporation was significantly less.

Reproducibility of the method. The reproducibility of the MLC method was examined by determining the response of the same four allogeneic combinations on three separate occasions at approximately weekly intervals. The results in Table II showed that the MLC response in suspension cultures was reproducible.

Discussion. The design of the above-described method was prompted by observations made during a study comparing the magnitude of 150 allogeneic responses, using a recently reported method of MLC (4). In the latter, mixtures of 1 and 2 × 10^5 R cells and 2, 4, and 6 × 10^5 S cells were incubated in

TABLE II. Reproducibility of the MLC Response.[a]

Combination			
ACx	BCx	CAx	CBx
cpm/culture			
30,302	32,998	39,685	30,157
30,101	29,994	34,307	24,020
32,181	35,611	37,464	25,553

[a] The MLC response of the four same allogeneic combinations of R and S lymphocytes was determined at approximately weekly intervals. Each suspension culture contained 1 × 10^5 R and 10 × 10^5 S lymphocytes. The response in autologous controls has been subtracted from the response in allogeneic cultures.

16 × 125 mm capped tubes placed in a CO_2 atmosphere. It was found that in several *strong* responses no difference was noted between ^{3}H-thymidine incorporation in cultures of 4 and 6 × 10^5 S cells to 2 × 10^5 R cells, suggesting that the cultures contained too many cells to determine the true magnitude of the response. In occasional *low* combinations no response occurred when 2 or 4 × 10^5 S cells stimulated 1 × 10^5 R cells, but a good response was observed when the same combinations were tested in cultures containing 2 × 10^5 R cells, indicating that 1 × 10^5 was too low a number of R cells to determine the magnitude of the response. An optimal method of MLC would measure all magnitudes of response in a constant number of R and S lymphocytes. In the present report a method is offered which comes closer to the optimal situation and which will discriminate better MLC responses of different magnitude than methods using settled cells.

The combination of suspending the lymphocytes, of increasing the numbers of S lymphocytes and of stabilizing the pH of the medium with the buffer Hepes increased the sensitivity of the quantitative MLC method, previously reported from our laboratory (4) 6 times. Our results finally showed that in the present method addition of 30% plasma to the medium and the use of 4 μCi of ^{3}H-thymidine were required to obtain a maximum response.

Summary. A new quantitative method of mixed lymphocyte culture using suspension

cultures of human lymphocytes is described. Compared to methods using settled cells, the new method increased the MLC response on the average 6 times permitting determination of a wider spectrum of MLC differences and a clearer discrimination between MLC responses of different magnitude.

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