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The Multiple Mixed Lymphocyte Reaction: Variables Important in the Test as a Measure of Lymphocyte Competence in Man[1,2]

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In order to utilize the mixed lymphocyte reaction (MLR) as an assay of T-lymphocyte competence, pools of target lymphocytes obtained from different individuals are used to increase the magnitude and decrease the variation of the *in vitro* response. We evaluated variations in MLR response due to variations in target cell populations. Response increased with an increased target/responder cell ratio. Peak response occurred with a target/responder cell ratio of between 1:1 and 1:4. Response to a pool of lymphocytes from different individuals increased as the number of individuals contributing to the pool increased. Peak stimulation occurred with three to four different donors to the target cell pool. Stimulation produced by pooled target cells resulted in a higher mean index of stimulation and decreased variation of response as compared to stimulation produced by target cells from individual donors. Stimulation produced by pooled target cells was approximately equal to the sum of the stimulation produced by each of the target cell populations acting alone. These findings indicate a practical method of modifying the MLR as a test of T-lymphocyte function.

When lymphocytes of two genetically dissimilar individuals are cultured together *in vitro*, the thymic derived T-lymphocytes of each individual respond to the histocompatibility antigens on the surface of lymphocytes of the other individual. This mixed lymphocyte reaction (MLR) is the basis of a current assay of histocompatibility (1–3). Responder lymphocytes are cultured with irradiated or mitomycin-C treated target lymphocytes, causing a one-way MLR. The degree of response is an indication of the histoincompatibility of the target tissue antigens. In addition, the MLR combines two properties that make this test a unique *in vitro* assay for T-lymphocyte competence. The MLR is a specific immunological response to cell surface antigens, but does not require prior sensitization to these antigens (4–6). These properties provide distinct advantages over conventional assays of lymphocyte competence such as lymphocyte response to nonspecific mitogens (phytohemagglutinin), which is not immunologically specific, and lymphocyte response to soluble protein antigens such as tuberculin which does require prior sensitization with specific antigen. In order to realize the potential of this test as a measure of T-lymphocyte competence, careful definition and, if possible, standardization is required. To this end, we and others have previously reported that pools of irradiated target lymphocytes obtained from several different individuals increase the magnitude and decrease the variation of the MLR response (7–10). We have further reported the use of pools of irradiated target lymphocytes to evaluate immunocompetence of individuals during disease states and during immunosup-

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pressive therapy (11–13). The present report details the rationale for the use of pools of irradiated target lymphocytes in a mixed lymphocyte assay of T cell function. In order to standardize the MLR as a test of immunocompetence, variations in response due to variations in target cell populations were evaluated. We investigated (1) variation in MLR response due to variation in concentration of irradiated target lymphocytes; (2) variation in MLR response produced by lymphocytes obtained from different donors of target cells; (3) the MLR response produced by pools of target lymphocytes obtained from several different donors.

MATERIALS AND METHODS

Cell preparation. Blood was obtained by venipuncture from normal human volunteers and anticoagulated with heparin (20 units heparin/ml, Upjohn, Kalamazoo, Michigan). Partially pure (90–98%) lymphocyte preparations were made by a simple centrifugation method previously described (14). Blood was centrifuged at 50*g* for 15 min and the upper two-thirds of plasma were removed with a pipet. The cells were washed and resuspended in Roswell Park Memorial Institute (RPMI) Medium 1640 with 15% pooled fresh homologous plasma, 50 units/ml penicillin, 50 g/ml streptomycin, and 300 g/ml glutamine (GIBCO, Grand Island, New York).

Irradiation of target lymphocytes. Lymphocytes to be used for stimulating cells were irradiated with 2000 rads of ionizing radiation (Siemens Stabilipan 250 unit with a 2-mm aluminum filter, utilizing 250 kV and 15 mA, and delivering 500 rads/min).

Cell culture system. Cells were pipeted into triplicate sets of 16 × 150 mm sterile glass test tubes with stainless steel closures. MLRs were performed with 5×10^5 responding lymphocytes. The concentration of irradiated target lymphocytes was varied. The total final volume of each culture was 1.5 ml. The tubes were incubated for 7 days at 5°C from the horizontal in a 5% CO_2 95% air humid atmosphere at 37°C.

Assay procedure. After 7 days of incubation, 0.5 ml of RPMI Medium 1640 containing 2 Ci of tritiated thymidine (sp act 1.9 Ci/mmole, Schwartz-Mann, Orangeburg, New York) was added to each tube. Four hours later the cultures were terminated with 8 ml of cold saline. Samples were washed twice with 5% trichloracetic acid and twice with absolute methanol. Samples were then assayed for incorporation of tritiated thymidine as previously described (15).

The mean and standard deviation of each triplicate culture was determined. The significance of the difference between two means was calculated by Student's *t* test. Data are reported as counts per minute (cpm). (Standard deviation of triplicate cultures ranged from 5 to 15% of the mean.)

Variation of target cell population. For each experiment the responder population consisted of 5×10^5 lymphocytes obtained from a single individual. The target cells were varied in three ways: (1) the number of irradiated target cells was varied to obtain a target responder cell ratio that ranged from 1/256 to 8/1; (2) several MLRs were performed by stimulating one responder population with target cells obtained from different individuals; (3) target cells from several different individuals were irradiated and then pooled prior to introduction into the culture system.

RESULTS

Effect of varying the target cell concentration. Stimulation of a constant number of responder cells (5×10^5) increased as target cell concentration was increased

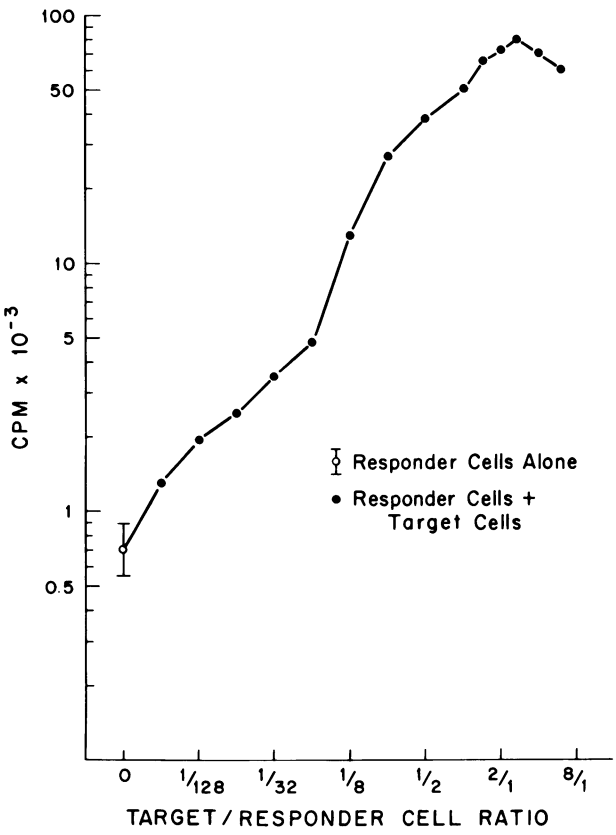

FIG. 1. Effect of varying target cell concentration on the MLR. A constant responder cell population (5×10^5 cells) is stimulated by increasing concentrations of irradiated target cells. The target/responder cell ratio is varied from 1/256 to 6/1.

(Fig. 1). Significant stimulation ($P < .05$) was first noted with a target/responder cell ratio of 1/32. In 23 experiments the minimum number of target cells causing significant stimulation ($P < .05$) ranged from 2 to 3×10^4 (target/responder cell) ratio of 1/32 to 1/16. The response varied with the target cell concentration over a wide range (1/16 to 1/1 target/responder cell ratio). In 25 experiments maximum stimulation occurred with a target/responder cell ratio of 1/1 to 4/1 ($5–20 \times 10^5$ target cells). Higher concentration of target cells resulted in either a plateau or a decrease in the response.

MLR response with different target cell populations. The responses of a single responder population to target lymphocytes obtained from four individuals is depicted in Fig. 2. A family of dose–response curves with approximately parallel slopes was produced. This finding was noted in ten experiments each performed with a single responder population exposed to four separate target cell populations. The source of responder cells was different in each experiment. For each responder population a family of similar curves was produced by different target cell stimuli. Because the target/responder cell ratio that causes peak stimulation varies between target cell populations and because the degree of response noted at maximum stimulation also varies between target cell populations, parallel dose–response curves are not appreciated if one looks only at the 1/1 to 4/1 target/responder cell range usually used for MLR histocompatibility testing.

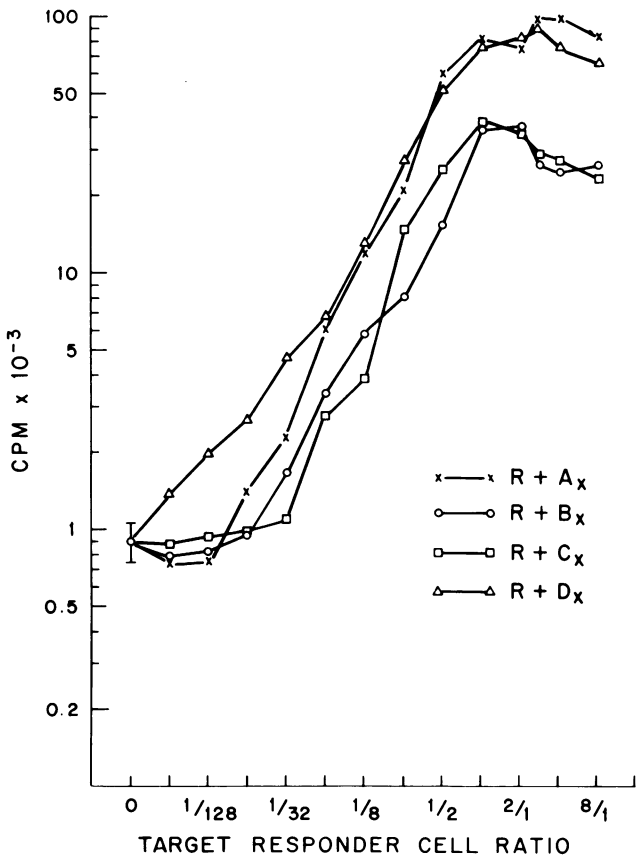

FIG. 2. MLR response to different target cell populations. A constant responder cell population (5×10^5 cells) is stimulated by varying concentrations of irradiated target cells (Ax, Bx, Cx, Dx) obtained from four individuals A,B,C, and D.

Effect of pooling target lymphocytes from several individuals. A single responder population was stimulated with a pool of target cells derived from several individuals. The responder cell number (5×10^5) and the total target cell number (1×10^6) (and the target/responder cell ratio (2/1) were held constant. The results of three such experiments are shown in Fig. 3. In each experiment a different responder cell population (R_1, R_2, R_3) was cultured with irradiated lymphocytes from different donors of target cells. As the number of individuals contributing cells to the target cell pool was increased, the response increased. Peak stimulation occurred with from three to four different donors of target cells. Additional target cell populations caused either a plateau or a fall in response.

Comparison of the stimulation produced by pooled vs individual target cells. To compare stimulation produced by target cells acting alone or in concert with allogenic cells, a comparison of stimulation of a single responding cell population to pooled vs individual target cells was made (Fig. 4). Because a pool of target cells from four donors usually caused maximum stimulation, this number was chosen for comparison to single MLRs. A wide range of target/responder cell ratios was used. At each concentration stimulation produced by a pool of four target cell populations was equal to or greater than that produced by each target cell population acting alone. To determine whether an equal number of cells from an individual donor

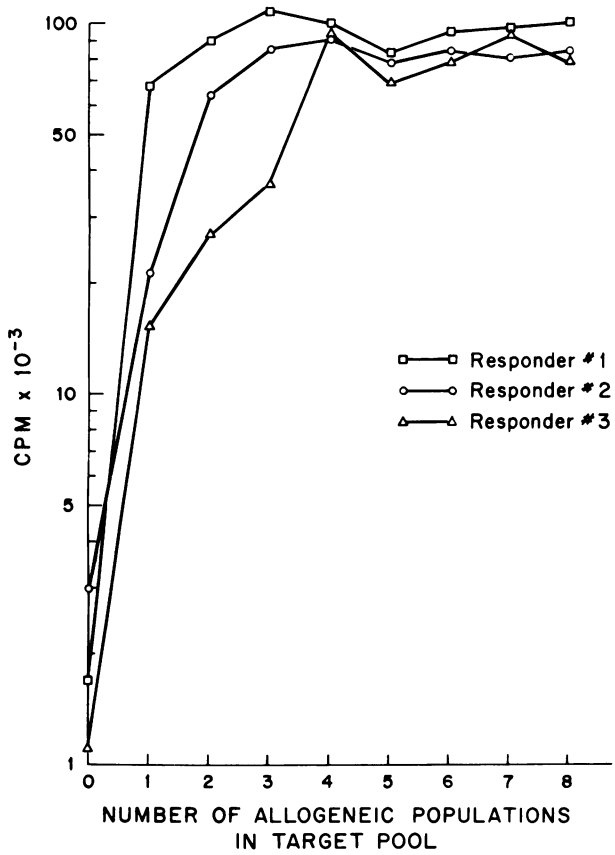

FIG. 3. MLR stimulation produced by a pool of target lymphocytes. Three responder populations R_1, R_2, and R_3 (5×10^5 cells each) were each stimulated with a pool of target lymphocytes obtained from several individuals. The total target cell concentration (1×10^6) was held constant. The number of different individuals contributing cells to the target cell pool was varied from 1 to 8.

could stimulate responder cells as well as an equal number of cells pooled from various donors, a comparison of stimulation produced by pooled vs individual target cells at maximum stimulatory concentration (1×10^6 cells) was performed. Eleven experiments with different responder and target cell populations are summarized in Table 1. In 8 of 11 experiments the pooled cells were more stimulatory than any of the individual cell populations. Evaluation of index of stimulation [cpm of (Responder Plus Target Cells)/(Responder Cells Alone)] produced by pooled and individual cell populations reveals (1) a slightly higher mean index of stimulation with the pooled (120) vs the individual (106) target cell populations; (2) a tenfold decrease in variation of response with the pooled (range 34–315) vs the individual (range 2.5–435) target cells; (3) the indices of stimulation produced by 13 of 44 (30%) reactions with individual cell populations were below the lowest index of stimulation noted with the pooled cells (Fig. 5). Thus, although stimulation produced by pooled cells does not greatly increase the mean index of stimulation, it does eliminate the lower range of values produced by individual target cell populations. This elimination of low degrees of stimulation due to weak antigenic disparity is essential for the use of the MLR as a screening assay of lymphocyte competence.

Independence of stimulation produced by pooled target lymphocytes. In order to

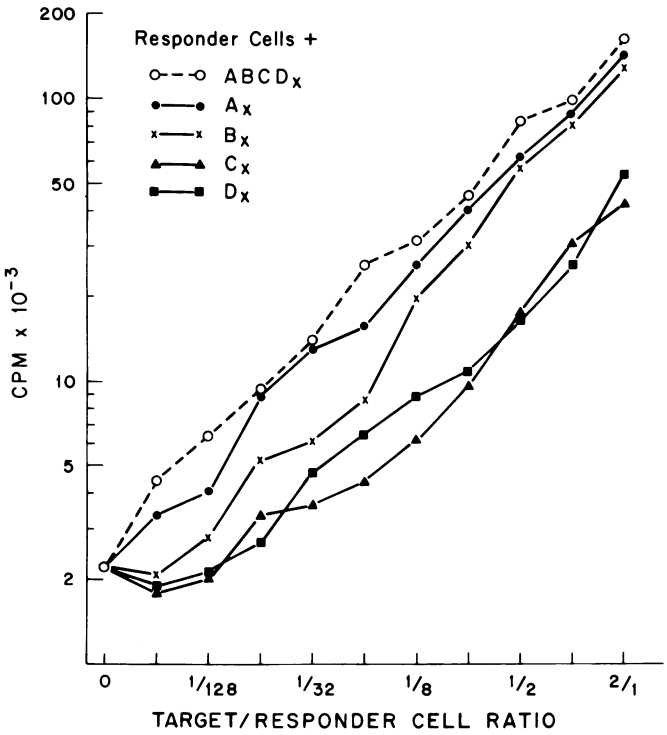

FIG. 4. Comparison of MLR stimulation produced by pooled vs individual lymphocyte populations. A constant responder population (5×10^5 cells) was stimulated by irradiated cells (Ax, Bx, Cx, Dx) obtained from four individuals A,B,C, and D. The responder population was also stimulated by a pool of lymphocytes obtained from these same donors (ABCDx).

evaluate the mechanism of increased stimulation noted by pooled lymphocytes from different donors of target cells, the response curve to pooled cells obtained in Fig. 4 was compared to a curve constructed by adding the responses obtained from four separate cultures of nonpooled cells (Fig. 6). The response to pooled cells was approximately equal to the sum of individual responses to each target cell population alone. For example, response to a pool of 1×10^6 irradiated cells ($\times$ = irradiated cells) from individuals A, B, C, and D (ABCDx) is equal to the sum of responses to each component of the pool (R + 1×10^6 (ABCDx)) = (R + 2.5×10^5 Ax) + (R + 2.5×10^5 Bx) + (R + 2.5×10^5 Cx) + (R + 2.5×10^5 Dx). This relationship is not valid at concentrations of target cells giving maximum stimulation; the maximum response to pooled target cells is less than the sum of the maximum responses to the individual target cell donors. These findings suggest that within a single culture system the response to target cells from an individual donor is specific, and is independent of the simultaneous response to other target cell populations present in the culture.

Comparison of stimulation produced by different pools of target cells. To determine the variability of stimulation produced by a pool of target cells from four different donors picked at random we compared the stimulation produced by two such sets of target lymphocytes.

In each experiment a single responder population (5×10^5 cells) was separately stimulated by two sets of target cells (ABCDx and EFGHx) each composed of equal

TABLE 1
Comparison of Pooled and Individual Mixed Leukocyte Reactions CPM × 10^{-3} in Cultures Containing:

Experiment[a] number	Responder cells alone (5×10^5) R	Responder cells and irradiated target cells[b] (1×10^6) Pooled MLR R + (ABCDx)	Individual MLR R + Ax	R + Bx	R + Cx	R + Dx
1.	.17	54	35	18	17	23
2.	6.3	216	16	126	117	130
3.	.5	33	30	29	22	29
4.	2.2	150	43	55	143	134
5.	.45	95	63	43	24	76
6.	.43	65	65	57	101	58
7.	.53	100	159	129	95	20
8.	.51	63	207	144	96	102
9.	3.0	119	73	43	63	53
10.	3.0	220	136	173	204	119
11.	2.0	97	11	28	64	90
Index of stimulation[c]		mean 120 range (34–315)			106 (2.5–435)	

[a]Each experiment was performed with a different responder cell population and pooled target lymphocytes obtained from different donors.

[b]Ax-irradiated target lymphocytes (1×10^6 cells), (ABCDx)-pool of irradiated target lymphocytes (2.5×10^5Ax+2.5×10^5Bx+2.5×10^5Cx+2.5×10^5Dx Cells). The cpm of irradiated cells cultured alone were subtracted from the cpm of each MLR. There was no difference in the cpm of the pooled and nonpooled irradiated cells cultured alone.

[c]Index of stimulation = (responder cells + target cells)/responder cells alone.

TABLE 2
Comparison of Pooled MLR Response to Two Sets of Target Cells CPM × 10^{-3} in Cultures Containing:

Experiment[a] number	Responder cells alone (5×10^5)	Responder cells and 1×10^6 (ABCDx)[b]	Responder cells and 1×10^6 (EFGHx)
1.	2.4	55	129
2.	.7	78	89[c]
3.	1.2	40	122
4.	.3	57	93
5.	1.0	50	89
6.	.5	95	56
7.	1.5	100	63
8.	1.7	42	62
9.	3.5	65	63[c]
10.	2.5	82	100[c]
11.	.9	86	66[c]
12.	3.0	87	70[c]
13.	1.2	119	220
14.		106	49

[a]Each experiment was performed with a different responder cell population and pooled target lymphocytes obtained from different donors.

[b](ABCDx) is a pool of irradiated lymphocytes from individuals A, B, C, and D. (EFGHx) is a pool of irradiated lymphocytes from four other individuals E, F, G, and H.

[c]Difference in response to two lymphocyte pools not significant at the $P = .05$ level of confidence.

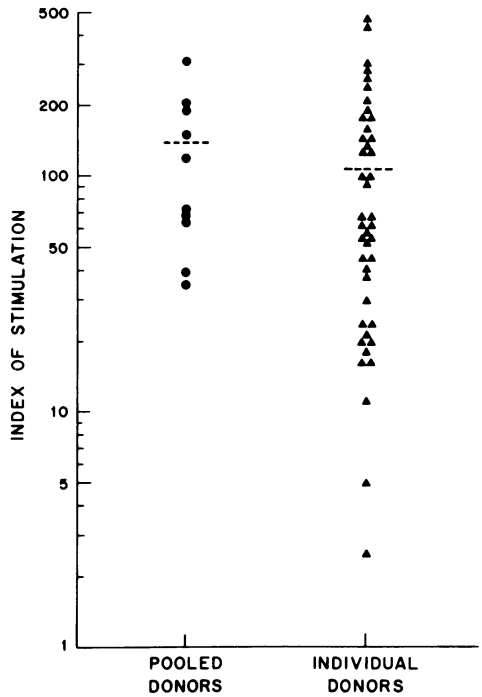

FIG. 5. Comparison of indices of stimulation produced by pooled and individual target cell populations. Each point represents the index of stimulation (responder + target cells)/(responder cells alone) of a different responder cell population (5×10^5 cells) stimulated by either pooled or individual target cell populations (1×10^6 cells).

numbers (2.5×10^5) of cells from four unrelated individuals (Table 2). In each of 14 experiments a different responder and different sets of target cells were used. In 5 of 14 experiments the difference in the stimulation produced by the two pools was not statistically significant at the $P < .05$ level. In 11 of 14 experiments the variation in response to the two target cell pools was less than twofold. In none of the experiments was the variation greater than threefold.

DISCUSSION

The findings reported here suggest essential modifications to ensure that the mixed lymphocyte reaction can be a practical *in vitro* assay of lymphocyte competence. Although the MLR is widely used as a test of histocompatibility (2, 3), its value as a test of lymphocyte competence has not been fully assessed. The MLR is an immunological response that is antigen specific, yet does not require prior sensitization to the homologous cell surface antigens that elicit the response. This characteristic sets the response apart from both PHA stimulation which is not antigen specific and soluble protein antigen stimulation which requires prior antigenic sensitization of the responding lymphocytes. In order to utilize these unique properties and standardize the assay one must overcome the variation in stimulation of normal lymphocytes produced by variation in the cell surface antigens of different allogenic cell populations. It is also necessary to evaluate variations in the reaction due to variation in target cell concentration. In order to increase response by the simultaneous stimulation of more than one clone of antigen specific responder

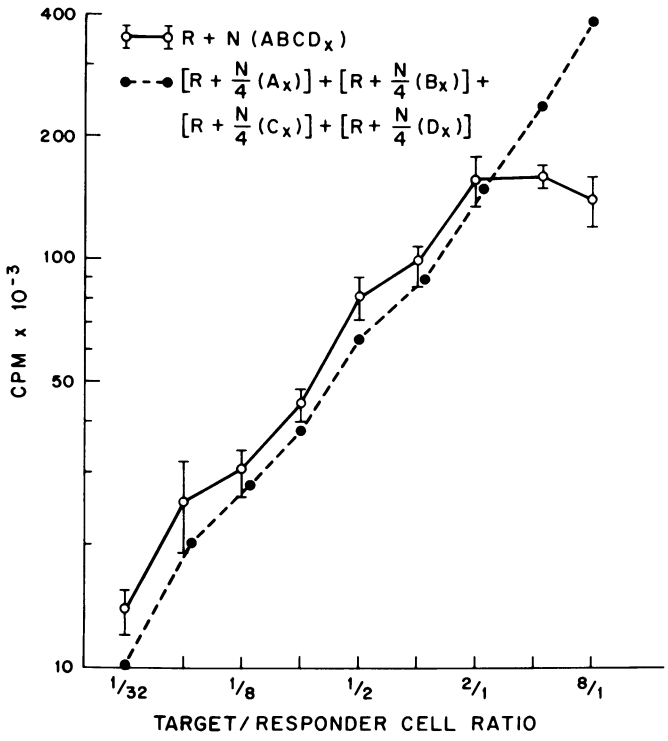

FIG. 6. Comparison of stimulation produced by a pool of target lymphocytes to the sum of stimulation produced by these target cells acting independently. (This figure is constructed from Fig. 4.) A constant responder population (R) (5×10^5 cells) was stimulated by four lymphocyte populations acting alone and in combination in a target cell pool. Response to the pooled target cells R + N (ABCDx) is compared to a curve constructed by the addition of responses to the individual target cell populations acting alone (R + N/4 (Ax)) + (R + N/4 (Bx)) + (R + N/r (Cx)) + (R + N/4 (Dx)). Equal target cell populations are compared. Thus response to 1×10^6 (ABCDx) is compared to response to (2.5×10^5 Ax) + (2.5×10^5 Bx) + (2.5×10^5 Cx) + (2.5×10^5 Dx).

lymphocytes, it is necessary to evaluate stimulation produced by pooled target lymphocytes from different individuals.

By varying the target cell concentration we observed a definite dose-response curve. This relationship was most apparent in the suboptimum range of target cell concentration. This constant dose-response relationship is even more apparent when one examines the family of similar curves produced by different target cell populations each stimulating the same responder population. (Fig. 2). These findings suggest that for complete comparison of antigenic disparity between a responder population and different target cell populations response should be measured at both suboptimal and maximum stimulating concentrations of target cells.

Combination of several allogenic lymphocyte populations into a pool caused greater stimulation and less variability than single MLRs. Of greater importance, the lowest index of stimulation produced by pooled target cells was higher than that produced by 30% of the individual target cell populations (Fig. 5). Thus, stimulation with an individual target cell population may produce low MLR response due to low degree of antigenic disparity. This might be misconstrued as responder lymphocyte incompetence. Pooled cell stimulation avoids low MLR response by providing antigens from several individuals, thus stimulating several clones of responder

lymphocytes. Osoba and Falk (9) have noted even less variation of response to pools of target cells with different major HLA antigens.

Stimulation of pooled target cells was equal to the sum of responses to each individual donor in the pool. This finding which is similar to the additive response to isoantigens noted with rat lymphocytes (5) and the additive response to soluble protein antigens noted with human lymphocytes (16) supports a clonal hypothesis in which distinct clones of lymphocytes respond independently to different antigens (6, 17). Similar findings have been reported using lymphocytes from two strains of rats (18).

Han and Pauly (19) recently reported that pooled target cell stimulation is synergistic rather than additive. The response to pooled cells was compared to the *mean* response of individual target cell stimulation R + (1.5 ml ABCx) vs [(R + 1.5 ml Ax)/3] + [(R + 1.5 ml Bx)/3] + [(R + 1.5 Cx)/3]. These authors assume that changes in target cell concentration cause a linear change in MLR response over the concentration range that they are evaluating and that the slope of that dose–response curve is 45 degrees: 3 (R + .5 Ax) = (R + 1.5 Ax). Neither our data (Figs. 1, 2) nor that of other investigators (2, 16, 18) indicate this to be true, especially at higher target cell concentrations where MLR response forms a plateau. We have shown that the response to pooled cells must be compared to the actual responses to individual components of the pool: R = (1.5 ABCx) vs (R + .5 Ax) + (R + .5 Bx) + (R + .5 Cx). If this is done in the suboptimal target cell concentration ranges the response to pooled cells is seen to be additive rather than synergistic (Fig. 6).

Lack of summation of the response at high target cell concentration may be due to either exhaustion of nutrient capacity of the culture or competitive recruitment of indifferent lymphocytes by more than one clone of responding lymphocytes. While Wilson has provided strong evidence against recruitment of indifferent lymphocytes in the rat MLR (6), other investigators have noted blastogenic factors in human MLR culture supernatants which appear to stimulate indifferent homologous lymphocytes (20, 21). High dose inhibition might also represent a phenomenon similar to inhibition of lymphocyte stimulation by high concentrations of soluble protein antigens (22). This type of inhibition may be due to production of a soluble feedback inhibitor of cell mediated immunity (23).

Use of a pool of target lymphocytes taken from different individuals provides a practical method for utilizing the MLR as a screening test of lymphocyte competence. In order to use single MLRs as an assay of lymphocyte competence, it is necessary to stimulate a responder population with several target populations. This method will establish a range of response, the variation of which is due to variation in the histocompatibility of the target cell populations. Since response to each target cell population in a pool is independent of the response to other target cells present, the use of a target cell pool has a distinct advantage over performance of several MLRs with individual cell populations. The antigens present on the surface of each target cell population contribute to the total stimulation, thus increasing response and decreasing variation due to individual antigen disparity. As noted above, the important advantage of pooled lymphocyte stimulation is the elimination of low levels of response due to low antigenic disparity. In addition, a single reaction with a pool of target lymphocytes requires fewer responder lymphocytes than multiple cultures containing each of the target cell populations individually. Since the minimum response to any single target cell population occurs at a target/responder

cell ratio of about 1/32 to 1/16 and the maximum response occurs at a ratio of about 1/1 to 4/1, it is theoretically possible to use from between 16 and 128 different target cell populations in a pool, expecting an independent response to each one. The limiting factor is probably the number of different recognizable antigenic specificities present on the surface of human lymphocytes. This number is probably closer to 16 than 128 (6, 24, 25). However, our findings indicate that it is not necessary to use such a large number of target cell donors in a pool. Any four target cell populations picked at random and pooled will increase stimulation, decrease variation of response, and decrease the likelihood of low response due to weak antigenic disparity. Individuals whose lymphocytes yield low indices of stimulation by this screening assay can then be further tested for immunocompetence. Our experience indicates that T-lymphocyte response to a random pool of target cells correlate well with other measures of immunocompetence such as PHA and specific antigen stimulation (11). In addition, this test may be of specific diagnostic value for detection of rejection of transplanted organs (12). This approach indicates a practical method of utilizing the unique properties of the MLR as a screening test for lymphocyte competence.

SUMMARY

Since the MLR is a specific immunologic response that does not require prior sensitization to the foreign antigen, this *in vitro* assay is an ideal test for T-lymphocyte competence. In order to decrease variation in the test due to variation in histocompatibility differences, pools of target lymphocytes obtained from several different individuals have been used to decrease the variation and increase the magnitude of the MLR response. The present report details the kinetics of this pooled target cell MLR. Response is directly proportional to the number of target cells present, increasing to reach a maximum reaction at a target/responder cell ratio of between 1:1 and 1:4. The linear dose-response relationship is most apparent in the suboptimal concentration of target lymphocytes. Response to target lymphocytes increases as the number of genetically different individuals contributing to the target pool is increased. Peak response occurs with approximately three to four different donors of target cells. Stimulation produced by pools of target cells yields a higher index of stimulation and less variation than stimulation by target cells obtained from individual donors. The mechanism responsible for enhanced response to pooled target lymphocytes is due to independence of stimulation produced by the individual donors to the target pool. Therefore, the response is approximately equal to the sum of the responses to each of the individual target cell populations alone.

These findings indicate that MLR response to pools of target lymphocytes is a useful tool for testing T-lymphocyte competence. The MLR is an antigen specific reaction that does not require prior sensitization to the foreign antigen. The combination of different cell surface antigens in a pool of target lymphocytes decreases variation of response due to variation in histocompatibility. Since the concept of pooled target cells was first introduced in 1973 we, and other investigators have reported successful application of this technique for testing T-lymphocyte competence in a variety of disease states.

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