Mixed Leukocyte Culture with Mouse Spleen Cells in a Serum-Free Medium[1] (36545)

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The finding that allogeneic leukocytes obtained from human donors show transformation when they are cultured together (1, 2) suggested the application of this phenomenon to studies of cellular response to transplantation antigens in other species (3–7), usually with measurement of the incorporation of ^{3}H-thymidine into the transformed proliferating cells (2). The application of the mixed leukocyte culture (MLC)[3] to the mouse met with some difficulty because of the poor viability of the cells. However, because of the availability of defined inbred strains, this species is very useful for studies in transplantation immunology, and there have been a number of reports on the MLC in the mouse (8–13). These have described different methods and conditions for eliciting the response.

The present paper reports a demonstration of a response in MLC between two inbred strains of mice, which involves a serum-free medium. The use of this medium yielded ratios of incorporation which continued to rise over a longer period than in serum-containing medium, and attained higher levels.

Materials and Methods. Cells and tissue culture. CBA (H-2^k) and BALB/c (H-2^d) female mice 8–12 weeks old were obtained from The Jackson Laboratory, Bar Harbor, ME. Spleens were removed aseptically and teased. The suspended cells were passed through a stainless steel mesh (80 gauge), washed once with ice cold RPMI-1640 medium (4) (Grand Island Biological Co., Grand Island, NY 14072) containing heparin (10 μ/ml), penicillin (50 μ/ml), streptomycin (50 μg/ml), and supplemented with glutamine (L-glutamine, 200 m*M*, 1 ml/100 ml of medium, Flow Laboratories, Rockville, MD 20852).

Thymus cells were prepared in essentially the same way.

Bone marrow cells were prepared by aseptically removing the tibia and femur and flushing out the marrow with medium, using a 1-ml syringe and 27-gauge needle. Heparin was omitted from the second and third washes.

The cells were counted and resuspended in the same medium to give a final number of 3×10^6 cells of each strain/tube. The final volume in each tube was 3 ml. The cultures were set up in triplicate, in plastic culture tubes (Falcon Plastics, Los Angeles, CA 90045, Cat. No. 3033) which stood in a vertical position in a humidified 37° incubator containing 95% air and 5% CO_2. After 3 days, 1 ml of fresh medium was added to each tube. Tritiated thymidine (sp act 14 Ci/mmole; New England Nuclear, Boston, MA 02118) was added at 1 μCi in 1 ml of medium to each tube 18 hr before harvesting.

Harvesting. The cells were washed once with ice-cold phosphate-buffered saline solution and 3× with cold 5% trichloroacetic acid. The sediment was dissolved in Hyamine 10x, incubated for 60 min in a 56° water bath, and mixed with 10 ml of Bray's solution (14). The samples were counted in a

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[3] Abbreviations used in this paper: cpm, counts per minute; FCS, fetal calf serum; MLC, mixed leukocyte culture; POPOP, 1,4-bis[2-(4-methyl-5-phenyloaxazolyl)]-benzene; PPO, 2,5-diphenyloxazole; ^{3}H-thymidine, tritiated thymidine.

Packard Tricarb scintillation counter (Packard Instrument Co., Chicago, IL 60616) and all the results given are the mean values of triplicates expressed as counts per minute per tube (cpm). The ratio of incorporation was calculated by dividing the cpm of the mixed cultures by the sum of the cpm of the separate ones. Alternatively, the specimens for determining the uptake of ^{3}H-thymidine were prepared by filtration rather than centrifugation. In these experiments, each culture tube was centrifuged at 4° for 10 min at 1800 rpm, and the cell pellet was washed twice with cold saline solution. To the cell pellet in each tube 3 ml of cold TCA were added, and the tubes were incubated at 4° for 1 hr. The resulting precipitate was collected on Whatman filter paper no. 3 with suction, and washed 5 times with 3 ml 5% cold TCA, then 3 times with acetone (3-ml volumes). The filters were dried at 90° for 30 min, and the radioactivity was determined in 5 ml toluene scintillation fluid (3.8 liters of toluene, 22.7 g PPO, and 1.89 g POPOP) in the scintillation counter, rather than Bray's solution. All data presented are mean values from at least 3 experiments in each of which the triplicate cpm values varied by less than 15% from the mean value.

Results. Mixed leukocyte cultures with 5% fetal calf serum (FCS). In the first series of experiments, 3×10^6 CBA spleen cells were incubated with 3×10^6 BALB/c spleen cells. The medium contained 5% heat-inactivated FCS, and the cultures were harvested after 96 hr. The incorporation in the mixed cultures was almost always higher than the sum of the incorporation in the separate ones, but the ratio of these values was small and inconsistent, and the incorporation in the control cultures was fairly high. The overall viability of the lymphocytes, judged by trypan blue dye exclusion, was between 60 and 80%.

In this series of experiments, the inconstancy of the results, and the fact that the lowest ratios of incorporation were found among the experiments with the greatest level of incorporation in the separate cultures, suggested the possibility of a nonspecific stimulation of the cultures. For this reason the concentration of FCS was decreased progressively in the following experiments.

TABLE I. ^{3}H-Thymidine Incorporation in Mixed Spleen Cell Cultures.[a]

	Source of cells			
FCS %	CBA+ BALB (cpm)	CBA (cpm)	BALB (cpm)	Ratio of incorporation
5	6924	1949	799	2.5
4	7244	1818	729	2.7
3	7249	1204	599	4.0
2	4029	1419	429	2.1
1	3134	729	299	3.0
0	2029	94	49	14.0

[a] All data are mean values from at least 3 experiments, in each of which the individual cpm values varied by less than 15% from the mean value.

Comparison between mixed cultures grown in serum-free and serum-containing medium. Cultures were set up in which FCS was used in a range of concentrations from 5 to 1%, and zero. Table I shows the incorporation of ^{3}H-thymidine on termination of the cultures after 96 hr, the mean values obtained in 3 experiments. The control cultures of separate cells showed progressively less incorporation with progressive decrease in concentration of FCS, reaching a level, in the serum-free culture, of 6 and 5% of the levels found with 5% FCS, for BALB/c and CBA cells, respectively. The mixed leukocyte cultures also showed a level of incorporation which was fairly constant in the range of 5 to 3% FCS. Below this level the incorporation decreased with decreasing concentration of FCS, but because of the greater relative decrease of the individual, unstimulated, cultures, the ratio of incorporation increased to a level of 14 in the absence of FCS. The differences between 5 and 1% FCS are not regarded as meaningful.

Comparison between serum-containing and serum-free cultures at different periods of culture. In the first experiments on the time relations of the difference between the two kinds of culture, the incorporation was measured after 48, 72 and 96 hr. In cultures harvested after 48 hr, no significant differences in the ratios were found among the groups. There was a relatively high background incorporation in all, probably because

TABLE II. ^{3}H-Thymidine Incorporation in Mixed Spleen Cell Cultures With and Without FCS, at Various Time-Intervals.

FCS in medium %	Day:	Cpm, ratio of mixed culture to sum of unmixed							
		4		5		6		7	
5		7111/2896	(2.4)	3183/4197	(0.8)	298/621	(0.5)	265/1090	(0.2)
0		2779/276	(8.6)	2620/220	(11.8)	1527/97	(15.8)	714/163	(4.4)

of nonspecific uptake of the label by the cells other than the lymphocytes (15). In the cultures harvested at 72 and 96 hr, of this series of experiments, the ratio of incorporation increased to 8–9 in the serum-free cultures, but remained near 2 in the presence of 5% FCS. The absolute counts of the MLC in the serum-free culture increased only to about 2×, but a concomitant reduction in the incorporation of the separate cultures caused the increase in the ratio.

Cultures harvested after longer periods showed further decreases in the incorporation by the separate, unstimulated, cultures in the serum-free medium, with corresponding increases in the ratio of incorporation to a maximum of over 15 on day 6 (Table II). In contrast, there was a marked decrease in the ratios of incorporation in the cultures containing FCS, to a level below 1 by day 5. In a series of six such experiments, the optimal time for harvesting was found to be 6 days, in four of the experiments, and 5 days in the other two.

Spleen cell cultures harvested by filtration. Three experiments with serum-free medium were done by this method of collection. In these, in addition to the sets of control tubes with separate BALB/c or CBA cells each at 3×10^6 cells/tube, with the sum of the mean counts taken as the divisor for obtaining the MLC ratio, tubes with separate cells at 6×10^6 each were set up, and the arithmetic mean of these levels was used as the divisor. In the 3 experiments, with both sets of separate-cell controls, the lowest of the 6 ratios thus obtained was 12.2. The effect of serum in the medium was also observed in these experiments, two ratios obtained on day 6, in the presence of 5% FCS, being less than 3.

Cells of other sources in the MLC. On the basis of the above results, it was decided to examine the mixed leukocyte cultures with thymocytes and bone marrow cells. In preliminary experiments to establish a procedure, in the present system, for distinguishing between cells providing antigen and cells reacting with blastogenesis, an MLC was set up, as above, but with either the BALB/c or the CBA cells irradiated at 500 R before culturing, by analogy with the one-way MLC produced by Bach and Voynow (16), on treating cells of one of the sources with mitomycin. Three such experiments showed, in all cases, no incorporation by irradiated spleen cells, either separate or in an MLC. However, if MLC was carried out with cells of either strain irradiated, ^{3}H-thymidine incorporation was found, at a level greater than the incorporation in a separate culture of the unirradiated strain by a ratio between 3.5 and 4.3. These results were typical of a one-way MLC.

In the tests involving thymus and bone marrow cells, it was found that the thymus cells did not incorporate thymidine in either the presence or absence of FCS, but could serve as a source of allogeneic antigen, producing a "one-way" reaction, as shown in Table III. Table III also shows that bone marrow cells gave a significant mixed cell reaction, although to a lower degree than the spleen cells.

Discussion. The serum-free medium and the MLC. As indicated above, our earlier attempts to follow published procedures for carrying out the MLC with mouse spleen cells yielded results which were not sufficiently reproducible, an observation which has also been mentioned by Dutton (8). The use

TABLE III. ^{3}H-Thymidine Incorporation in Separate and Mixed Cultures of Thymus, Bone Marrow and Spleen Cells.

CBA	BALB	Cpm	Ratio
Thymus	—	0	
—	Thymus	0	
Thymus	Thymus	0	0
Spleen	—	632	
Spleen	Thymus	2950	4.7
—	Spleen	320	
Thymus	Spleen	2445	7.6
—	Bone marrow	270	
Thymus	Bone marrow	566	2.1
Bone marrow	—	269	
Bone marrow	Thymus	700	2.6

of homologous serum as a support for the medium was rejected because of the possibility of variable amounts of cell-membrane antigens which might be liberated from the white and red blood cells during the preparation of the serum (17, 18). Autologous serum, which has been used in rat MLC (19), would be difficult to obtain in adequate amounts in the mouse. In further work with the FCS we encountered the additional difficulty that the degree of stimulation of the cultures by the FCS was not proportional to the concentration. Different batches of FCS gave different degrees of stimulation, which were greater than the differences in cell viability, and which sometimes appeared to mask a specific effect (10). The lots of FCS that were available to us presumably acted as stimulants to the cells, as well as nutritional supplements.

The time curve of the uptake by the separate cell cultures, in the presence and absence of FCS, suggests the possibility that the effect of the FCS in this situation has a similar basis to that of heterologous antilymphocytic serum (ALS) (20). Fresh human serum gave us high background readings, in the same range as those given by FCS (unpublished observations). The high degree of incorporation reported by Adler *et al.* (9) may therefore be a summation of an effect such as that of ALS, combined with the specific stimulation of a mixed leukocyte reaction. In keeping with this suggestion is the point raised by Adler (9) that one should be aware of possible differences in degree of stimulation of individual human sera used in their medium, because of differences in titer of "anti-mouse lymphocyte globulin" in such sera. The present adaptation of the culture system to a serum-free medium obviates the considerations of such nonspecific stimulation. Our peak response, which was between days 5–6, is similar to what has been reported by others (10, 19).

The finding in this study that thymus cells did not incorporate thymidine under our culture system, but could serve as a good source of allogeneic antigen agrees with earlier observations of Chapman and Dutton (21). The observation that bone marrow cells are capable of undergoing blastogenesis, and thus incorporating ^{3}H-thymidine, is consistent with previous observations on the immunocompetence of this cell line, and its capability of initiating a graft versus host reaction (22–24).

Summary. Following experiments which suggested that apparent stimulation of mouse spleen cells by fetal calf serum (FCS) was masking specific blastogenic effects in the mixed leukocyte culture (MLC), cultures of CBA and BALB/c spleen cells were set up in RPMI-1640 medium, containing FCS at 5, 4, 3, 2, 1 and 0%. On termininating the cultures on day 4, the ratio of ^{3}H-thymidine incorporation between the mixed and the separate cultures was found to be considerably higher in absence of FCS than in the presence of FCS at any of the concentrations indicated. A series of timed cultures, without any FCS, showed a ratio between the incorporation of ^{3}H-thymidine in the mixed cultures and the sum of incorporation in cultures of separate cells which reached a maximum level of 11–15, on day 5 or 6. In tests with cells of other sources, it was found that thymus cells could serve as a source of allogeneic antigen, but could not incorporate thymidine, and that bone marrow cells could be stimulated, but to a lower degree than spleen cells.

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