Clin. exp. Immunol. (1970) **6**, 345–361.

ALLOGRAFT IMMUNITY *IN VITRO*

I. CULTIVATION CONDITIONS AND MIXED LYMPHOCYTE INTERACTION OF MOUSE PERIPHERAL LYMPHOCYTES

P. HÄYRY* AND V. DEFENDI

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania

(*Received* 29 *August* 1969)

SUMMARY

We have adapted mouse peripheral lymphocytes to culture as a preliminary step in designing a model for the study of allograft immunity *in vitro*. The isolation of peripheral leucocytes is facilitated by using Plasmagel® as an erythrocyte-agglutinating agent. The yield of leucocytes can be considerably increased by intravenous injection of the donor animals with supernatant fluid from *Bordetella pertussis* cultures and the lymphocytes thus mobilized react both to phytohaemagglutinin (PHA) and allogeneic stimulus, as do lymphocytes from untreated animals. Preparations which contain more than 25–50 RBC/WBC are refractory in the mixed lymphocyte interaction (MLI). The optimum cell density for the proliferative response is approximately $1–3 \times 10^6$ lymphocytes/ml. Various nutritive milieu were tested and found to have little influence on the MLI; both normal and suspension media behaved in a similar manner. PHA causes a vigorous proliferative response in mouse peripheral lymphocytes, the ^{3}H–TdR incorporation values in PHA-containing cultures at peak point of stimulation (3rd day) being up to 1000 times those observed for control cultures. The allogeneic response in the MLI takes place later (6th to 7th day) and is weaker, about one-tenth the PHA response, when strains differing at the *H*-2 locus are used as cell donors. Because the specific proliferative response to allogeneic stimulus in mixed culture, regardless of the way it is measured, is indistinguishable from the response produced by other non-specific factors, these other factors must be critically excluded. It appears that supplementing the culture medium with low concentrations of certain lots of foetal calf or agamma-newborn-calf serum permits the study of the specific response at an optimum sensitivity.

INTRODUCTION

The mixed lymphocyte interaction (MLI), that is, the induction of DNA synthesis and proliferation in mixed lymphocyte cultures from genetically dissimilar individuals (Bain,

Correspondence: Dr Vittorio Defendi, The Wistar Institute of Anatomy and Biology, 36th at Spruce Street, Philadelphia, Pennsylvania 19104, U.S.A.

* Present address: Departments of Pathology and Surgery, University of Helsinki, Helsinki, Finland.

C

Vas & Lowenstein, 1964; Hirschorn *et al.*, 1964), has become an important tool for probing, in an *in vitro* self-enclosed system, the events which occur during sensitization of lymphocytes to allogeneic transplantation antigens, and also for identifying tissue incompatibilities between donor and recipient (Amos & Bach, 1968; Albertini & Bach, 1968). In addition, it has been shown, especially in studies on inbred strains of rat, that the proliferative phase of the MLI possesses the characteristics required for an immunological phenomenon, that is, selectivity and specificity (Wilson, Silvers & Nowell, 1967; Schwarz, 1968).

The transplantation genetics of the laboratory mouse have been characterized up to the point where the individual antigenic pattern of the major locus (*H*-2), at least within certain alleles (*H*-2 $^{a, b, d \text{ and } k}$), is reasonably well known (Snell & Stimpfling, 1966). Several inbred mouse strains differing at defined histocompatibility loci have also been developed (Staats, 1968). Therefore, it appears that the ideal cell donor animal for quantitative and analytical studies of the cell-bound immune response on an *in vitro* level would be the mouse.

In reactions which attempt to measure the response of lymphoid cells to allogeneic transplantation antigens, like the normal lymphoid transfer reaction and graft-*versus*-host reaction, the capacity to respond is highest in lymphocytes derived from the peripheral blood (Hildemann, 1964; Wilson & Billingham, 1967). This seems also to be the case with the MLI (Wilson, 1967). Cultivation of the peripheral lymphocytes of the mouse, however, has been difficult and only a few attempts thus far have been successful. Tridente, Cappuzzo & Chieco-Bianchi, 1967; Festenstein, 1968; Main, Jones & Cole, 1969).

In the present communication, we wish to report our experience in the cultivation of mouse peripheral lymphocytes *in vitro* and our development of a highly reproducible technique for the MLI using strain combinations which differ at the major mouse histocompatibility locus.

MATERIALS AND METHODS

Animals

The following male inbred mouse strains were used: C57BL/6, CBA, BALB/c, B10.D2, DBA/2, A/Jax, C3H and (BALB/c+CBA) F_1 hybrids. The animals were either purchased from Jackson Laboratories, Bar Harbor, Maine, or bred in our own colony. Because of the difficulties connected with heart puncture in very small animals, adult mice weighing more than 25 g were preferred.

Chemicals, media and sera

Three lots of PHA-M (Difco Laboratories, Detroit, Michigan) were pooled using phosphate-buffered saline (PBS) as diluent, then redistributed to 1-ml ampoules and frozen. For each experiment one or two ampoules were removed from deep freeze, and after the experiment, the unused material was discarded. Colcemid® (Ciba Pharmaceuticals, Summit, New Jersey) was diluted into sterile, distilled water and stored at a concentration of 10 μg/ml at 4°C before use. The media were obtained as powdered or liquid concentrates from different commercial sources (Flow Laboratories, Rockville, Maryland; Microbiological Associates, Bethesda, Maryland; Grand Island Biological Co., Grand Island, New York) and were prepared according to the manufacturer's instructions and filter-sterilized. The sera were also purchased from various commercial sources (Flow Laboratories; Microbiological Associates; Baltimore Biological Laboratories, Cockeysville, Maryland;

Industrial Biological Laboratories, Rockville, Maryland; Reheis Chemical Co., Chicago, Illinois) with the exception of the horse, chicken and rabbit sera, which were a gift from Baltimore Biological Laboratories.

Leucocyte isolation

The method of Tridente *et al.* (1967) was employed. Blood was collected into a heparinized syringe. If not otherwise stated, blood from several animals of one inbred strain was pooled and mixed with an equal volume of Plasmagel® (Roger Bellon, Neuilly, France). The red blood cells were left to sediment and after 20–30 min at room temperature, the leucocytes could be recovered from the 'buffy coat' layer. Because of the possibility that mouse plasma and also Plasmagel might interfere with the proliferative reaction, the leucocytes were washed twice with tissue culture medium.

Leucocyte mobilization

Leucocytes isolated using Plasmagel yield approximately 2–4 $\times$ 10^6 WBC/mouse. The yield can be considerably increased by first injecting animals with pertussis vaccine or with supernatant fluid from *Bordetella pertussis* culture (Morse & Bray, 1969) (Table 1). Because of the economic advantages which this technique offers, pertussis-injected animals were used as cell donors in several experiments. The animals were injected intravenously, 3 days prior to bleeding, with 0·1 ml of a 1:4 diluted supernatant fluid from *B. pertussis* culture (Lot No. CS-XIX, gift from Dr Morse, Rockefeller University, New York).

Lysis of red cells

For some experiments it was necessary to obtain pure leucocyte suspensions, completely devoid of contaminating red cells. Erythrocytes were lysed by mixing two parts of the cell suspension with an aqueous solution of 0·83% ammonium chloride (Strander & Cantell, 1966). The suspension was incubated for 10 min at 4°C, centrifuged and washed twice with the culture medium. The resulting leucocyte suspension was completely free of red cells and had a viability of 99·8%, as determined by the dye exclusion test.

Assay and cultivation conditions

The total number of lymphocytes in a cell suspension was determined by diluting an aliquot into an aqueous solution of 2% acetic acid (1:10) and by performing differential counts with phase contrast microscopy. For the assay, the cells were usually adjusted to a concentration of 1·0–1·5 $\times$ 10^6 lymphocytes/ml; 0·5 ml of cell suspension of both strains or 1·0 ml of either strain (for control cultures) was distributed in sterile, 13-mm, loosely stoppered Wassermann tubes. For each determination, triplicate cultures were used. The culture medium, unless otherwise stated, consisted of double-strength Eagle's amino acids in Earle's balanced salt solution (2E + 1E), with freshly added glutamine and streptomycin (50 mg/100 ml)—penicillin (50,000 IU/100 ml). The medium was supplemented with various concentrations of different sera, most commonly with foetal calf serum. The tubes were incubated in an upright position in a humidified atmosphere of 5% CO_2, 10% O_2 and 85% N_2 (Festenstein, 1968) for different periods of time, and the cultures were pulsed in the warm room with 0·5–2 μCi of ^{3}H-TdR (Schwartz Biochemicals; specific activity, 6·0 mCi/mM), usually for a period of 16 hr, and harvested by placing the tubes in the cold. For experiments with PHA, PHA-M was added at varying concentrations to the medium at

the time the culture was initiated, and for each measurement, triplicate cultures, usually consisting of $1–3 \times 10^6$ lymphocytes/ml, and similar pulsing schedules were used.

Measurement of response

The response was determined by either of two methods: with the method most often used, the amount of ^{3}H-TdR incorporated into acid-insoluble material was determined by *liquid scintillation counting*. For processing the labelled DNA, a technique similar to that described by Wilson, Blyth & Nowell (1968) was used. The entire procedure took place at 4°C, all of the reagents being equally well chilled. The cells in the culture tubes were spun down and the supernatant discarded. A volume of 0·025-ml of 'carrier' (2% bovine serum albumin in 10% NaCl) and thereafter 3 ml of 5% trichloracetic acid (TCA) was added per tube by vigorous injection with an automatic pipette. The tubes were incubated for 30 min and then centrifuged. The precipitate was washed twice with TCA. After final washing all radioactivity had disappeared from the supernatant. The precipitate was dissolved at room temperature in 0·5 ml of 2% NH_4OH. A volume of 0·2–0·3 ml of solubilized material was spread on a filter pad (Reeve Angel, glass fibre filter No. 934AH) previously placed in a scintillation vial. The open vials were heated at 110°C for 1·5–2 hr and 5 ml of scintillation fluid (125 ml of Liquifluor®, New England Nuclear Corporation, Boston, Massachusetts, in 3 litres of toluene) were added. The radioactivity was counted immediately in a Beckmann Model No. 1050 liquid scintillation counter. No kemiluminescence ever appeared and 'blank' values for non-labelled controls did not differ from the background values of the counter. This technique had the advantage of being highly reproducible (the triplicate counts seldom differed more than 10% from each other) and counting could be performed immediately after the samples were processed.

In order to determine the amount of cells participating in the reaction, autoradiograms and labelled nuclei counts were done on several occasions. Smears were made on gelatinized microscope slides and fixed with methanol, and autoradiograms were performed using Kodak AK-10 stripping film. After 3–7 days' exposure, the autoradiograms were developed and stained with either May-Grünwald–Giemsa or Giemsa stain. For the labelled nuclei count, 500–1000 nuclei were scored.

Chromosome preparations

Colcemid (0·1 μg) was added to the culture tube for 2–3 hr. Thereafter, a technique described by Moorhead *et al.* (1960) was applied with slight modification (Häyry, Virolainen & Defendi, 1969). A volume of 0·5 ml of culture fluid was removed and 2 ml of distilled water added. The material was transferred to a 3-ml centrifuge tube; after hypotonic treatment for 12 min, it was spun down. Glacial acetic acid–methanol (1:3) was used as fixative. Slides were air-dried and stained with Giemsa stain.

RESULTS

Response of peripheral lymphocytes to PHA

PHA is a strong mitogenic agent for lymphocytes. In order to gain some preliminary experience with the isolation–cultivation conditions of the peripheral lymphocytes of the mouse, the PHA effect was first examined. Particular attention was given to: (1) variables in the culture conditions, such as the culture medium (spinner-medium *versus* regular

medium), source and amount of serum used to supplement the medium, cell density and PHA concentration; (2) the timing and magnitude of response; and (3) the 'background' ^{3}H-TdR incorporation in non-PHA-containing control cultures. In order to determine the relative numbers of PHA-responding cells in different mouse lymphoid cell populations, splenic and lymph node cells were tested in similar test conditions, as well.

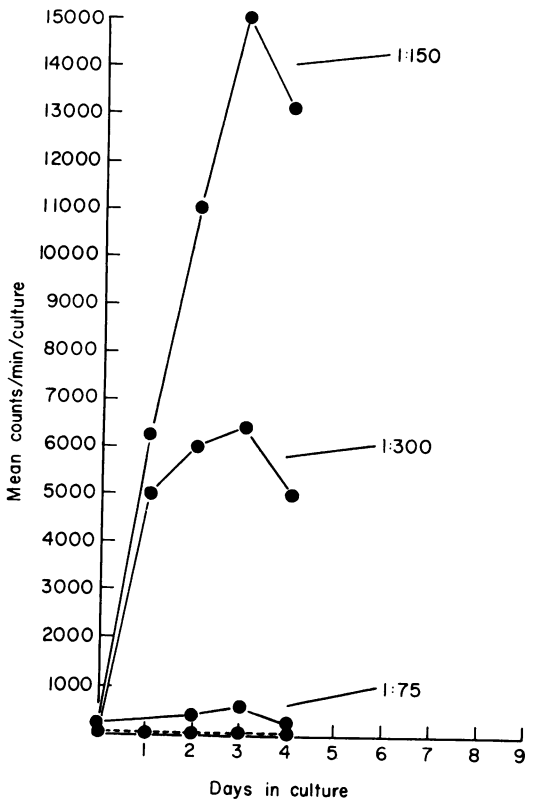

FIG. 1. Effect of PHA concentration on the ^{3}H-TdR incorporation in C57BL/6 peripheral lymphocytes (1×10^6 lymphocytes/culture). Cultures pulsed daily with 0·25 μCi/ml ^{3}H-TdR for 16 hr. ——, PHA-containing cultures; - - - -, control.

The results can be summarized as follows: The response to PHA in the presence of regular medium (2E+1E) was no better than it was in spinner-medium (2E+1E). The type of serum used to supplement the medium was of some importance. Calf sera (CS) were usually inferior to foetal calf sera (FCS) in supporting the response. When tested in 10% serum concentrations, the ^{3}H-TdR incorporation values in the presence of calf sera were only in the 30–80% range of values obtained with FCS. The incorporation values in control cultures were, on the other hand, somewhat higher in FCS- than in CS-containing cultures. The best responses were obtained using cell densities of $1–3 \times 10^6$ lymphocytes/ml, when 1 ml of cell suspension was used per tube. The peak of ^{3}H-TdR incorporation invariably took place during the 3rd day in culture. The magnitude of the response was dependent on the dilution of PHA; the best responses were always obtained using a 1:150 (final)

dilution of PHA-M (Fig. 1). At 72 hr in culture, after 2 hr of colchicine arrest, 5–15% of the cells were in mitosis; after a 16-hr labelling period, 60–90% of the nuclei carried the label. The PHA-induced stimulation of splenic and lymph node cells was lower than that of peripheral lymphocytes (Fig. 2).

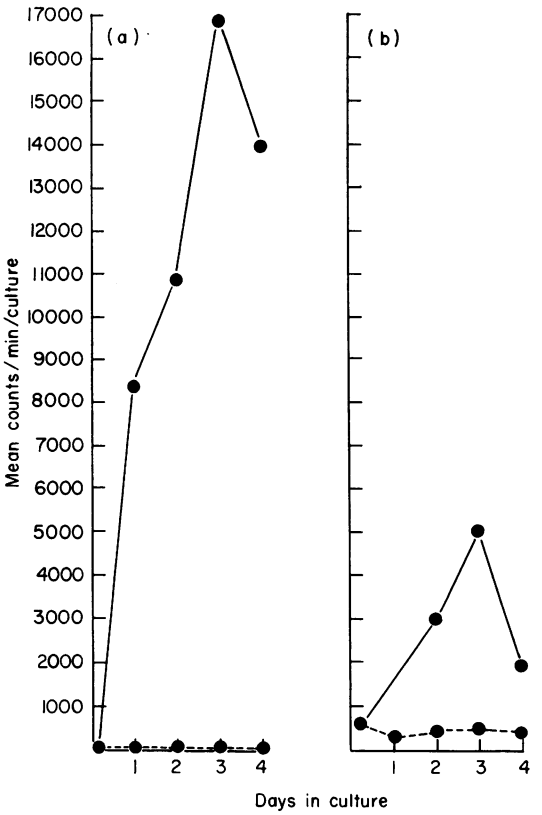

FIG. 2. Comparison of the PHA-response between C57BL/6 peripheral lymphocytes (1×10^6) (a) and splenic nucleated cells (3×10^6) (b). PHA-M 1:150. ——, PHA-containing cultures; – – – –, control.

Response of peripheral lymphocytes to allogeneic stimulus—timing of the response

With most species the maximum response in mixed cultures of peripheral lymphocytes, as measured by ^{3}H-TdR incorporation, takes place at 6–7 days in culture (Amos & Bach, 1968; Wilson, 1966, 1967). Because of the many variables being tested in some of the following experiments, it was necessary to know when the peak point of stimulation would be reached. This was determined by kinetic experiments in which cultures were pulsed for 16 hr and harvested daily. The cells were suspended in a concentration of 1×10^6 lymphocytes/ml in medium (2E+1E) supplemented with 8–10% of FCS. The FCS lot was selected according to the results obtained in PHA experiments—the serum lot which constantly gave the lowest incorporation values in non-PHA-containing control cultures was chosen.

It was observed that shaking or vigorous handling of the culture racks during the first

3 days of incubation often would result in a diminished response or no response. In several experiments with inbred strains differing in the major *H*-2 histocompatibility locus, the peak point of ^{3}H-TdR incorporation was seen to occur most frequently, despite some variation from experiment to experiment, on the seventh day; thereafter, incorporation values declined. A representative experiment of this kind is shown in Fig. 3.

Mixed cultures using cells from spleens and lymph nodes of the same strains of mice were also done in similar test conditions, although it was necessary to use 3×10^6, instead of 1×10^6, spleen cells. The ^{3}H-TdR incorporation values in mixed spleen cell cultures were much lower than in either mixed lymph node or peripheral lymphocyte cultures, with a broad ill-defined maximum response occurring between the 3rd and 6th days. The control incorporation values in non-mixed cultures of mouse spleen cells were high, and

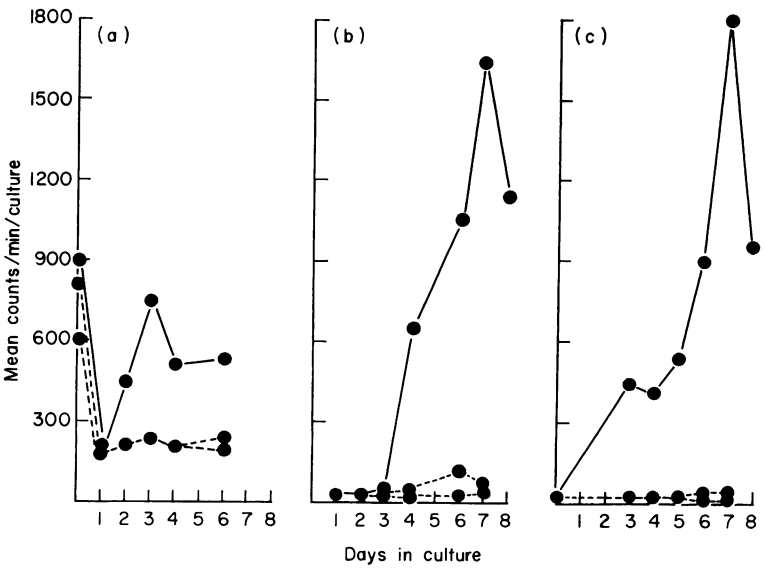

FIG. 3. ^{3}H-TdR incorporation in mixed cultures of C57BL/6 and CBA lymphocytes. Comparison of sources. (a) Spleen, (b) lymph node, and (c) peripheral blood. Cultures pulsed daily with 0·5 μCi/ml of ^{3}H-TdR. ——, Mixed cultures; – – – –, non-mixed cultures.

the 'stimulation index' (ratio of ^{3}H-TdR incorporation in mixed culture to mean incorporation in non-mixed cultures) remained low. The response with lymph node cells was considerably better. The maximum response with these cells, as with the peripheral lymphocytes, took place at the 6th to 7th day in culture, and the control incorporation values in non-mixed cultures were constantly low. One comparative experiment is shown in Fig. 3.

Effect of pertussis 'mobilization'

In the experiments described above, it was necessary to kill ten to fifty mice for a single experiment. However, it was found that the yield of leucocytes could be considerably increased by prior intravenous injection of pertussis vaccine or supernatant fluid from *B. pertussis* cultures (Morse & Bray, 1969; Main *et al.*, 1969) (Table 1). Lymphocytes were

obtained from pertussis-treated and from non-treated animals and their responses in MLI were compared; four inbred strains differing at the *H*-2 locus (A/Jax, C57BL/6, BALB/

TABLE 1. Yield of leucocytes from the peripheral blood of different strains of mice after pertussis mobilization and Plasmagel isolation

Strain	Pertussis*	No. of determinations	Yield of leucocytes/ animal ($\times 10^6$)	% granulocytes	Yield of lymphocytes/ animal ($\times 10^6$)
C57BL/6	No	4	4·9 ± 1·1†	13·0 ± 3·0	4·2 ± 1·2
CBA	No	4	2·2 ± 0·5	23·0 ± 2·0	1·7 ± 1·0
BALB/c	No	4	3·4 ± 1·5	24·0 ± 2·1	2·5 ± 1·6
C57BL/6	Yes	3	29·3 ± 1·5	31·0 ± 6·0	20·6 ± 3·0
CBA	Yes	7	22·1 ± 2·4	26·4 ± 1·9	15·8 ± 1·6
BALB/c	Yes	7	40·1 ± 4·3	22·7 ± 2·2	29·0 ± 2·3
B10.D2	Yes	3	21·3 ± 3·8	26·0 ± 3·5	16·0 ± 2·9
DBA/2	Yes	3	39·0 ± 14·8	33·5 ± 7·4	28·0 ± 12·7
A	Yes	2	16·5 ± 1·0	26·0 ± 2·1	13·0 ± 0·7
C3H	Yes	3	27·0 ± 5·8	25·0 ± 2·6	20·6 ± 5·1

* 0·1 ml of 1:4 diluted (PBS) supernatant fluid from *B. pertussis* culture (i.v.) given 3 days previously.
† Standard deviation.

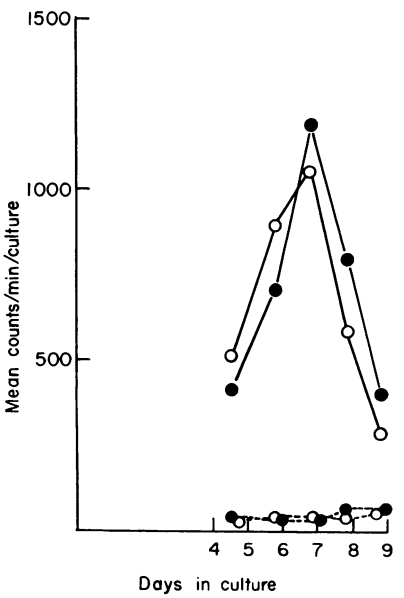

FIG. 4. ^{3}H-TdR incorporation in cultures from pertussis-treated (●) and non-treated donors (○). Strain combination BALB/c and CBA. Cultures pulsed with 0·5 μCi/ml of ^{3}H-TdR daily. ——, Mixed cultures; – – – –, mean values of non-mixed cultures.

and CBA) were used as cell donors. No qualitative or quantitative differences in this respect in the response were observed (Fig. 4). Because of this fact, in most of the experiments to be described, the pertussis-mobilized lymphocytes were used.

Effect of purification and red blood cell contamination

Because of the possibility that the presence of red blood cells (RBC) might modify the MLI, the effect of contaminating red cells on the allogeneic response was also tested. Some red cells were always left in the isolate after Plasmagel sedimentation (approximately 8–15 RBC/WBC without pertussis mobilization and 0·5–5·0 RBC/WBC with pertussis). Therefore, the residual RBCs from the Plasmagel-isolated suspensions were lysed by ammonium chloride treatment and the ^{3}H-TdR incorporation values of peripheral lymphocytes treated with NH_4Cl were compared to those of untreated cells. In other experiments, Plasmagel-isolated lymphocytes were artificially mixed with mouse red cells, using a relatively pure washed red cell population. For the assay, the red cells were added from the same strain of origin of the lymphocytes; otherwise mixed cultures consisted of red cells from both strains, and non-mixed cultures from the strain of the donor only.

TABLE 2. The effect of the RBC/WBC ratio on the MLI (BALB/c + CBA)

RBC/WBC*	Mean counts/min/culture			Stimulation index†
	BALB + CBA	BALB	CBA	
2–3‡	2100	150	59	20·1
25	2110	120	100	19·2
50	1780	82	85	21·4
250	75	39	46	1·7
500	48	59	40	0·9

* RBC added according to WBC histocompatibility.

† Ratio of counts/min in mixed culture to mean counts/min in non-mixed cultures.

‡ Residual RBC left after Plasmagel isolation.

Lysis of the residual RBC from the reaction mixture did not enhance the MLI. However, artificial contamination of the lymphocyte cultures with red cells had a uniform and clear-cut effect. Invariably when red cells were assayed at concentrations of higher than 25–50 RBC/WBC, the MLI was either impaired or suppressed. A representative experiment, in which the addition of 250 or more RBC/WBC completely abolished the reaction, is shown in Table 2.

Effect of lymphocyte density

In some preliminary experiments with pertussis-mobilized leucocytes, the number of nucleated cells, rather than the number of lymphocytes, was used as the criterion of cell density for the assay. In these experiments, deviations between repeated tests were frequent and they were thought to be due to differences in the final lymphocyte densities in cell mixtures (Table 1). Therefore, in order to test the exact effect of cell density, experiments were performed in which varying numbers of lymphocytes (and unknown numbers of granulocytes) were suspended in 1 ml of culture medium. Triplicate cultures were harvested at 7th day in culture after a ^{3}H-TdR pulse for 16 hr.

The lymphocyte density was found to have a definite and reproducible effect on the

reaction. In present test conditions using cell densities lower than $0{\cdot}75 \times 10^6$ lymphocytes/ml, the ^{3}H-TdR incorporation in mixed cultures remained low. A diminished response was also observed when the cultures were crowded with more than $2–3 \times 10^6$ lymphocytes/ml. It was also noted that incorporation values of non-mixed controls tended to increase at densities higher than $1{\cdot}5 \times 10^6$ lymphocytes/ml (Fig. 5).

Effect of medium

The medium most commonly used for experiments of this kind is 'minimum essential medium' (MEM), consisting of the essential amino acids (Eagle, 1955) and a balanced salt solution. However, some investigators prefer spinner-medium, made specifically for suspension culture by omitting the bivalent cations from solution (Amos & Bach, 1968;

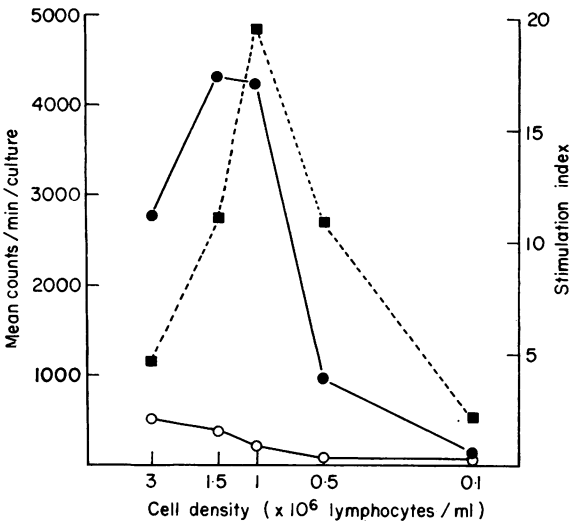

FIG. 5. Effect of lymphocyte density on the MLI. Strain combination BALB/c and CBS. Cultures pulsed with 1 μCi/ml of ^{3}H-TdR on the 7th day. ●, Mixed cultures; ○, mean value of non-mixed cultures; ■, stimulation index.

Main *et al.*, 1969; Manson *et al.*, 1969). We compared the effects of two lots of spinner-medium and five different lots of non-spinner, regular medium, all obtained from different commercial sources, in otherwise identical test conditions. The cultures were pulsed and harvested on the 7th day.

The results of these experiments may be summarized as follows. Varying the source and lot of medium, within the conditions tested, did not affect the magnitude of the response. Nor was any change noted when the balanced salt solution was supplemented with a one or two times concentration of the essential amino acids (1E+1E or 2E+1E). The exclusion of bivalent cations from the medium had no effect.

Effect of serum

Preliminary experiments indicated that both the source and concentration of the serum used to supplement the culture medium were of critical importance in successfully stimulating the MLI. In further experiments the serum effect was investigated more thoroughly.

Particular attention was given to: (1) the magnitude of the response, (2) the timing of the response, and (3) the background ^{3}H-TdR incorporation in non-mixed control cultures.

The effect of the *serum source* on the response was tested by comparing horse, lamb, rabbit, chicken and several individual lots of both calf and foetal calf sera at 10% concentrations. The conditions of culture were otherwise identical and cultures were harvested on the 6th to 7th day after a pulse of 24 hr.

TABLE 3. The effect of different sera and individual serum lots on the MLI (C57BL/6 and CBA)

Serum*	Mean counts/min/culture			Stimulation index†
	C57 + CBA	C57BL/6	CBA	
Lamb	1182	400	389	3·0
FCS No. 1	2860	1118	1150	2·5
FCS No. 2	1020	321	661	2·1
FCS No. 3	1800	128	132	13·9
FCS No. 4	1880	350	418	4·9
FCS No. 5	1960	1120	1240	1·7
CS No. 1	2300	300	350	7·1
CS No. 2	152	58	90	2·1
CS No. 3	83	65	62	1·3
CS No. 4	46	67	97	0·6
Horse	360	300	280	1·2
Rabbit	78	75	76	1·0
Chicken	82	70	74	1·1

* FCS, foetal calf serum; CS, calf serum. All sera 10% concentration.
† Ratio of counts/min in mixed culture to mean counts/min in non-mixed cultures.

According to the results of these experiments, the sera can be grouped in two categories (Table 3). The first group of sera, including all the foetal calf sera and one lot of calf serum (CS No. 66377), showed consistently high incorporation values in the mixed cultures; however, the values in control cultures were also relatively high. The other group of sera, including the other calf sera and the rabbit and chicken sera, did not support the reaction. Incorporation values in mixed cultures were low and cell viability, tested by the dye exclusion method, was also poor. The single lot of horse serum fell somewhere between the two groups.

The effect of *serum concentration* was investigated by separately assaying several different serum lots for each of three kinds of serum. Three lots of calf serum, four lots of foetal calf serum and two lots of agamma sera (one agamma horse and one agamma newborn calf) were tested.

All *foetal calf sera* supported the allogeneic reaction over a wide range of concentrations (3–20%). At higher or lower concentrations the ^{3}H-TdR incorporation was suppressed. Cell viability was good at FCS concentrations between 3 and 40% (in mixed cultures, at the 7th day of culture approximately 30–50% of the original inoculum), but sharply

declined in concentrations falling below this range. At high concentrations of FCS, the ^{3}H-TdR incorporation in control cultures was high, often close to the level of the values for mixed cultures. The best stimulation indexes (Table 4) were, therefore, obtained at an FCS concentration between 3 and 8%. When the response was measured by autoradiography, up to 15% of the nuclei were shown to carry the label after a pulse of 16 hr at the peak point of response.

TABLE 4. The effect of foetal calf serum* on the MLI (BALB/c + CBA)

Serum concentration (%)	Mean counts/min/culture			Cell viability†			Stimulation index‡
	BALB/c +CBA	BALB/c	CBA	BALB/c +CBA	BALB/c	CBA	
40	2060	580	880	35	10	15	2·7
20	3060	325	2400	30	12	20	2·3
10	3490	54	390	31	6	3	15·8
8	4650	19	109	38	4	10	72·8
5	3280	19	139	28	5	16	43·1
3	980	3	32	30	2	12	57·2
1	12	1	2	6	8	6	12·0

* Lot: Flow No. 455196.
† Determined by trypan blue uptake. Viable cells × 10^4. Original inoculum 1·5 × 10^6 lymphocytes/tube.
‡ Ratio of counts/min in mixed culture to mean counts/min in non-mixed cultures.

TABLE 5. The effect of calf serum* on the MLI (BALB/c + CBA)

Serum concentration (%)	Mean counts/min/culture			Stimulation index†
	BALB/c + CBA	BALB/c	CBA	
20	112	78	110	3·1
10	90	60	61	1·5
8	100	45	55	2·0
5	21	10	10	2·0
3	25	13	11	2·0
1	19	21	9	1·3

* Lot: Flow No. 421188.
† Ratio of counts/min in mixed culture to mean counts/min in non-mixed cultures.

Individual lots of *calf sera* were assayed in a similar way (Table 5), but with very different results. All the lots of calf sera but one were unable to support the MLI at any concentration; however, the values for ^{3}H-TdR incorporation in both mixed and non-mixed cultures remained low and close to each other. The one exception, lot CS No. 55377 (see Table 3), behaved approximately the same as the FCS.

The *agamma sera* behaved, in mixed cultures, in the same way as the FCS. At a concentration range of about 3–8%, they supported the reaction well, the agamma newborn

calf serum being about five times better than the agamma horse serum. However, the ^{3}H-TdR incorporation values in non-mixed control cultures remained low over the entire range of concentration. A typical experiment is illustrated in Table 6.

Our previous experiences with *mouse serum* have indicated that in concentrations higher

TABLE 6. The effect of agamma newborn calf serum* on the MLI (BALB/c + CBA)

Serum concentration (%)	Mean counts/min/culture			Stimulation index‡
	BALB/c + CBA	BALB/c	CBA	
40	18	13	42	0·7
20	3	13	11	0·2
10	1066	27	51	27·2
5	4200	29	184	39·6
3	5000	18	69	113·8
1	5	35	120	0·1

* Lot: BBL No. 9041181.

† Ratio of counts/min in mixed culture to mean counts/min in control cultures.

TABLE 7. The effect of mouse serum on the MLI (BALB/c + CBA)

Mouse serum†	8% FCS*				1% FCS*			
	Mean counts/min/culture			Stimulation index§	Mean counts/min/culture			Stimulation index§
	BALB/c + CBA	BALB/c	CBA		BALB/c + CBA	BALB/c	CBA	
None	1021	25	38	31·9	13	26	10	0·7
Fresh								
4%	531	17	128	7·5	32	19	5	2·4
2%	727	17	20	36·0	21	26	10	1·2
1%	501	35	25	16·6	25	11	10	2·5
Inactivated‡								
4%	156	20	18	7·9	6	13	24	0·3
2%	1039	21	32	38·8	26	23	5	1·8
1%	723	34	67	14·5	20	6	6	3·3

* FCS, Lot Flow No. 455196.

† BALB/c and CBA serum mixed in equal amounts.

‡ At 56°C for 30 min.

§ Counts/min in mixed culture per mean counts/min in non-mixed cultures.

than 5% this serum is generally toxic to the cultures. On the other hand, in concentrations of less than 2% the mouse serum is not able to support the viability of mouse peripheral lymphocytes. Because of this, the effect of mouse serum on the MLI was tested in the presence of FCS in two concentrations—an 8% concentration that supported the reaction

well, and a 1% concentration that failed to support it. Both fresh and heat-inactivated mouse sera were tested over the 1–4% range of concentrations. Sera pooled from both cell donors were used in these experiments.

Results of a representative experiment are presented in Table 7. When the culture medium was supplemented with 8% of FCS, neither fresh nor inactivated mouse serum at any concentration had an enhancing effect on the reaction. In fact, the incorporation values in the presence of mouse serum often remained under those observed in cultures containing only FCS. When the culture medium was supplemented with only 1% of FCS, the addition of mouse serum also failed to enhance the reaction.

Reproducibility of the response

To provide information concerning the uniformity of the response in various individuals of the same inbred strain against a common pool of allogeneic cells, leucocytes were collected separately from individual BALB/c mice. To ensure that the response, as measured by ^{3}H-TdR incorporation per culture, was totally due to the BALB/c cells, these were assayed against a suspension of (BALB/c+CBA) F_1 hybrid cells pooled from two to three mice. In order to note, also, possible changes in the timing of the response, triplicate cultures were pulsed and harvested daily.

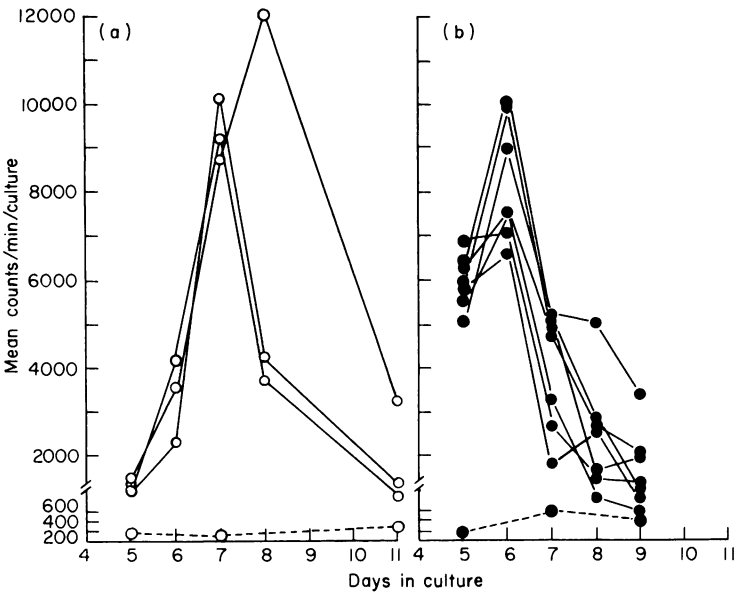

FIG. 6. Reproducibility of the MLI. Response of peripheral lymphocytes from individual BALB/c donors to (BALB/c + CBA) F_1 lymphocytes in two separate experiments. Cultures pulsed daily with 1 μCi/ml of ^{3}H-TdR. ——, mixed cultures; – – – –, mean incorporation in non-mixed cultures.

In two separate experiments, shown in Fig. 6(a) and (b), where cells from three and seven animals, respectively, of the responding strain (BALB/c) were individually tested against the hybrid, slight differences were observed in the timing of the maximum response, both between and within experiments. In experiment (a), the cells from two of the animals

responded identically while those from the third reached maximum ^{3}H-TdR incorporation 1 day later. In experiment (b), where test conditions were similar except for the lot of FCS used, the responses of separate individuals were essentially the same, but the time of overall maximum incorporation in this experiment was reached 1 day earlier than in experiment (a).

DISCUSSION

The response of mouse peripheral lymphocytes to PHA seems to be similar to responses to PHA observed with lymphocytes from other species (for references, see Elves, 1966). The maximum ^{3}H-TdR incorporation is observed at the 3rd day in culture, when the majority of lymphocytes, as determined by autoradiography and metaphase counts, seem to participate in the reaction. The observation that an optimum concentration of PHA is required for maximum stimulation corresponds to observations made with human (Wilson, 1966) and rat (Wilson, 1967) lymphocytes. However, quantitative comparisons between different species of the magnitude of the response and the optimum PHA concentration are difficult to make because of the different culture conditions used. The optimum dilution of PHA for mouse peripheral lymphocytes (1:150, PHA-M) for our culture conditions corresponds to that previously observed by Festenstein (1967). The combined treatment of PHA and Colcemid (Moorhead *et al.*, 1960) also makes possible repeated karyological analyses of this genetically important experimental animal (Häyry *et al.*, 1969).

The proliferative response of antigenically dissimilar lymphocytes in mixed culture has the essential characteristics of an immunological phenomenon; it is assumed this response represents the initial phase of cellular immune response to allogeneic transplantation antigens (Wilson *et al.*, 1968; Bach *et al.*, 1969). The behaviour of mouse peripheral lymphocytes in mixed culture seems to be similar to that of human and rat cells (Wilson, 1967; Amos & Bach, 1968); when the proliferative response is measured as ^{3}H-TdR incorporation, the peak of the response is observed at about the 6th to 7th day in culture, whereafter the incorporation values decline. The discrepancies between the timing of the response in our experiments and in those of other investigators using mouse lymphoid cells obtained from different sources (Dutton, 1965; Manson *et al.*, 1969) can be explained by differences in the experimental design. Thus, splenic lymphocytes seem to respond far less to allogeneic stimulus than do peripheral lymphocytes; also the number of contaminating red cells in the cell mixture (see also Main *et al.*, 1969), and the source and concentration of serum may greatly modify the reaction.

As pointed out before, lymphocytes in culture are able to respond by proliferation to a great variety of antigens and other factors. Some of these require the prior sensitization of the donor animal (for references, see Elves, 1966; Wilson & Billingham, 1967). In test conditions where a direct demonstration of the specificity of the proliferative reaction is not possible, e.g. by demonstrating that cells sensitized against one set of histocompatibility antigens react exclusively against cells possessing the same antigenic pattern (Häyry & Defendi, 1969), it is necessary to exclude all other factors which might contribute to a morphologically similar but non-related response in culture. It appears that some sera, at least in high concentrations, when supplemented to the culture medium, although being capable of supporting the viability and the proliferative interaction of lymphocytes in mixed culture, cause high responses in non-mixed control cultures. Such was the case with high, but not medium or low, concentrations of all foetal calf sera. Agammaglobulinaemic sera, al-

though able to support the MLI in a fairly broad range of concentrations, did not cause any response in non-mixed cultures, not even at high concentrations. Whatever non-specific proliferative factors are involved, from a practical point of view, these can be eliminated or excluded by the selection of a suitable serum lot and concentration.

Little is known about the leucocytosis-producing factor present in *B. pertussis* vaccine or culture fluid. Apparently, leucocytosis in the presence of this substance is not an immunological phenomenon, does not harm the test animal and can be produced repeatedly (Morse & Bray, 1969). Our studies, as well as those reported by others (Main *et al.*, 1969) indicate that cells obtained from pertussis-treated and non-treated animals react similarly in MLI. However, the possibility that this factor might act as an immunogen must be kept in mind, particularly if repeated bleedings of the same animal are required.

In some preliminary comparative testings between the leucocytosis induced by commercially available lots of *B. pertussis* vaccine and by the supernatant fluid (Häyry and Defendi, unpublished observations) it was found that a much stronger effect was obtained with the latter: the efficiency of different lots of vaccine varied considerably and with some of the lots was negligible. This observation is in accordance with that of Morse's (Morse & Bray, 1969) that the leucocytosis-producing factor(s) appear in larger quantities in the culture supernatant fluid.

The feasibility of supporting a standard MLI reaction characterized by a low background level in immunogenetically well-identified mouse peripheral lymphocytes permits the examination of several important questions in regard to the cell-bound immune response to foreign histocompatibility antigens *in vitro*, such as, whether it is possible to detect histocompatibility antigens determined by minor loci in this way; whether there are differences among the individual antigens regulated by the *H*-2 locus in their ability to induce the response; and whether cells in the MLI reaction ultimately become effector cells, capable of immunospecifically destroying allogeneic target cells in culture. Some of these questions are now under investigation.

ACKNOWLEDGMENTS

This investigation was supported in part by United States Public Health Service Contract PH43-65-1028 from the Viral Carcinogenesis Branch, National Cancer Institute and American Cancer Society Grant PRA-47. One of the authors (P.H.) is the recipient of a grant from the Finnish Medical Council, Helsinki, Finland and a Fulbright-Hays Travel Grant.

The authors are indebted to Miss Dolores Rago and Miss Catharine Consalvi for skilful technical assistance.

REFERENCES

ALBERTINI, R.J. and BACH, F.H. (1968) Quantitative assay of antigenic disparity at HL-A—the major histocompatibility locus in man. *J. exp. Med.* **128,** 639.

AMOS, D.B. & BACH, F.H. (1968) Phenotypic expressions of the major histocompatibility locus in man (HL-A): Leukocyte antigens and mixed leukocyte culture reactivity. *J. exp. Med.* **128,** 623.

BACH, F.H., BOCK, H., GRAUPNER, K., DAY, E. & KLOSTERMANN, H. (1969) Cell kinetic studies in mixed leukocyte cultures: An *in vitro* model of homograft reactivity. *Proc. nat. Acad. Sci. (Wash.),* **62,** 377.

BAIN, B., VAS, M. & LOWENSTEIN, L. (1964) The development of large immature mononuclear cells in mixed leukocyte cultures. *Blood,* **23,** 108.

DUTTON, R.W. (1965) Further studies of the stimulation of DNA synthesis in cultures of spleen cell suspensions by homologous cells in inbred strains of mice and rats. *J. exp. Med.* **122,** 759.

EAGLE, H. (1955) Nutrition needs of mammalian cells in tissue culture. *Science*, **122,** 501.

ELVES, M.W. (1966) *The Lymphocyte*. Lloyd-Luke (Medical Books), London.

FESTENSTEIN, H. (1968) Mouse peripheral-blood lymphocytes in culture. *Lancet*, **i,** 182.

HÄYRY, P. & DEFENDI, V. (1969) Allograft immunity *in vitro*. II. Production of effector cells in mixed lymphocyte interaction. *Proc. 4th Leucocyte Culture Conf.* (In press).

HÄYRY, P., VIROLAINEN, M. & DEFENDI, V. (1969) Method for chromosome preparations from mouse peripheral blood. *Proc. Soc. exp. Biol. (N.Y.)* (In press).

HILDEMANN, W.H. (1964) Immunological properties of small blood lymphocytes in the graft-versus-host reaction in mice. *Transplantation*, **2,** 38.

HIRSCHHORN, K., BACH, F., RAPAPORT, F., CONVERSE, J. & LAWRENCE, H.S. (1964) The relationship of *in vitro* lymphocyte compatibility to homograft sensitivity in man. *Ann. N.Y. Acad. Sci.* **120,** 303.

MAIN, R.K., JONES, M.J. & COLE, L.T. (1969) The mixed leucocyte reaction adopted to inbred mouse strains. *Proc. 3rd Leucocyte Culture Conf* (Ed. by W. O. Ricke). Appleton-Century-Crofts, New York.

MANSON, L.A., SIMMONS, T., MILLS, L. & FRIEDMAN, T. (1969) *In vitro* induction of homograft immunity. Detection by an antibody plaque reduction assay. *Proc. 4th Leucocyte Culture Conf.* (In press).

MOORHEAD, P.S., NOWELL, P.C., MELLMAN, W.J., BATTIPS, D.M. & HUNGERFORD, D.A. (1960) Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell Res.* **20,** 613.

MORSE, S.I. & BRAY, K.K. (1969) The occurrence and properties of leukocytosis and lymphocytosis—stimulating material in the supernatant fluids of *Bordella pertussis* cultures. *J. exp. Med.* **129,** 523.

SCHWARZ, M.R. (1968) The mixed lymphocyte reaction: An *in vitro* test for tolerance. *J. exp. Med.* **127,** 879.

SNELL, G.D. & STIMPFLING, J.H. (1966) Genetics of tissue transplantation. *Biology of the Laboratory Mouse* (Ed. by E. L. Green), p. 457. McGraw-Hill, New York.

STAATS, J. (1968) Standardized nomenclature for inbred strains of mice: Fourth listing. *Cancer Res.* **28,** 391.

STRANDER, H. & CANTELL, K. (1966) Production of interferon by human leukocytes *in vitro*. *Ann. Med. exp. Fenn.* **44,** 265.

TRIDENTE, G., CAPPUZZO, G.M. & CHIECO-BIANCHI, L. (1967) Lymphocyte interaction in mixed mouse leukocyte cultures (a preliminary report). *Histocompatibility Testing* (Ed. by E. S. Curtoni, P. L. Matting and R. M. Tosi), p. 67. Williams & Wilkins, Baltimore.

WILSON, D.B. (1966) Analysis of some of the variables associated with the proliferative response of human lymphoid cells in culture. *J. exp. Zool.* **162,** 161.

WILSON, D.B. (1967) Quantitative studies on the mixed lymphocyte interaction in rats. I. Conditions and parameters of response. *J. exp. Med.* **126,** 625.

WILSON, D.B. & BILLINGHAM, R.E. (1967) Lymphocytes and transplantation immunity. *Advanc. Immunol.* **7,** 184.

WILSON, D.B., BLYTH, J.L. & NOWELL, P.C. (1968) Quantitative studies on the mixed lymphocyte interaction in rats. III. Kinetics of the response. *J. exp. Med.* **128,** 1157.

WILSON, D.B., SILVERS, W.K. & NOWELL, P.C. (1967) Quantitative studies on the mixed lymphocyte interaction in rats. II. Relationship of the proliferative response to the immunologic status of the donors. *J. exp. Med.* **126,** 655.