Method for Chromosome Preparation from Mouse Peripheral Lymphocytes[1] (34534)

P. HAYRY,[2] M. VIROLAINEN, AND V. DEFENDI[3]
(Introduced by D. Kritchevsky)

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104; and III Department of Pathology, University of Helsinki, Helsinki, Finland

Since Moorhead *et al.* (1) described a simple method to obtain chromosome preparations from human peripheral blood by combining phytohemagglutinin (PHA) as a mitogenic agent with the air-drying techniques of Rothfels and Siminovitch (2), this technique has been applied to various mammalian species. Although the karyotype of the mouse is not particularly suitable for detailed karyologic examination, the well-known genetics of the animal, the availability of several inbred strains, and the existence of certain chromosome markers within a single strain, make cytogenetic analysis of the mouse a useful tool. The methods available, however, present considerable difficulties, particularly if repeated sampling from the same animal is required. Thus, the available techniques necessitate: (i) injecting the mouse with colchicine, followed by sacrifice of the animal (3); (ii) obtaining samples from bone marrow and searching for cells in mitosis; and (iii) culturing of a skin biopsy (4). Unlike those of most mammalian species, cultures of mouse peripheral blood have been unsatisfactory (5, 6), and the occasional successes seem to have been limited to only certain strains of mice (7). This difficulty has led investigators to use rather cumbersome methods, like placing small amounts of blood inside a Millipore chamber, which is then implanted into the peritoneal cavity of another animal (5, 8). Even in the few cases where a notable amount of metaphase plates have been obtained *in vitro* with techniques using peripheral blood (8), the response to the mitogenic agent within some strains seemed to be poor and the cultures seemed to degenerate during the second day in culture, that is, 1 day before the maximal blastogenic response occurs.

We report a method for chromosome preparations from mouse peripheral blood that requires only simple tissue culture facilities and that gives large numbers of analyzable metaphase plates per animal without need of sacrificing it. This method seems to be applicable to all common mouse strains.

Materials and Methods. Mouse strains. The following strains were used: A/J, DBA/2, C3H, C57BL/6, BALB/c, CBA, CBAT6T6 and F_1 (BALB/c + CBA) hybrids. The animals were either bred in our colony or obtained from Jackson Laboratories, Bar Harbor, Maine. Because of the technical difficulties connected with heart puncture of very small animals, adult mice weighing at least 20–25 g were preferred.

Collection and separation of peripheral leukocytes. Blood was collected through heart or orbital plexus puncture into a heparinized syringe or by bleeding from the tip of the tail into a heparinized tube. One-half ml of blood could be collected from a single mouse without losing the animal. The red cells were aggregated and separated by sedimentation with equal volumes of Plasmagel® (Roger Bellon, Neuilly, France) either in the syringe used for collection or in a thin, sterile test

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tube (9, 10). After 20–30 min of incubation in an upright position at room temperature, the red cells were well sedimented and the leukocyte-rich plasma could be collected. The red cell contamination rate was less than 10 RBC/WBC. The cells were washed twice with the culture medium. The number of nucleated cells was counted under a phase-contrast microscope using 2% acetic acid as diluent (1:10). In most cases the yield was approximately 1–2 $\times$ 10^6 nucleated cells/0.5 ml of blood.

Pertussis vaccine stimulation. The yield of peripheral leukocytes could be substantially increased by injecting the animal intravenously 3 days prior to the bleeding with 0.2–0.3 ml of *B. pertussis* vaccine (Eli Lilly & Co., Indianapolis, Ind.) or with 0.1 ml of 1:4 diluted supernatant fluid from *B. pertussis* culture (11). The animals survived this treatment well. The pertussis treatment increased the yield of leukocytes 10- to 20-fold and these cells responded as well to PHA as did leukocytes from untreated animals.

Culture conditions. The culture medium consisted of double-strength Eagle's essential amino acids in Earle's balanced salt solution (Flow Laboratories, Rockville, Md.; all-liquid concentrate), supplemented with 10% fetal calf serum (Flow) and streptomycin (100 μg/ml)-penicillin (100 IU/ml). Cultivation of 1–1.5 $\times$ 10^6 leukocytes was carried out in 1 ml of culture medium in 13 $\times$ 100 mm, loosely stoppered, Wassermann tubes in an upright position in a humified atmosphere of 5% CO_2 and air at 37°. PHA (PHA-M, Difco Laboratories, Detroit, Mich.) was added to the medium at the initiation of the cultures.

Estimation of the proliferative response. Two different techniques were used to estimate the proliferative response. First, the cultures were pulsed for 16 hr with 1 μCi of ^{3}H-TdR (Schwarz BioResearch, Orangeburg, N. Y.; sp act, 6.0 mCi/mmole), and the ^{3}H-TdR incorporation into acid-insoluble fractions was measured using conventional liquid scintillation counting techniques. Secondly, smears were made on gelatin-coated microscope slides and the proliferative response was measured using stripping film (Kodak-AR-10) autoradiography and by the percentage count of labeled cells.

Chromosome preparations. At about hours 70-72 of culture, 0.1 μg of Colcemid® (Ciba Pharmaceuticals, Summit, N.J.) was added per tube. The stock concentrate of Colcemid was prepared in distilled water (1.0 μg/ml), sterilized by filtration, and stored at 4°. Tubes were harvested 1-1.5 hr later. One-half of the culture medium was removed and replaced with 2 ml of distilled water and the tubes were gently shaken. After 10 min of hypotonic treatment, the cells were centrifuged at 1000 rpm using 3-ml conical centrifuge tubes. Thereafter the procedure of Moorhead *et al.* (1) was followed. Culture medium was removed and replaced with 2 ml of fixative (1 part glacial acetic acid in 3 parts of methanol) without disturbing the pellet. After 1 to 2-min incubation at room temperature, the pellet was gently disrupted by aspirating repeatedly with a Pasteur pipette. The cells were centrifuged and 2 ml of fresh fixative were added. This operation was repeated two times. The cells were resuspended in the fixative and a small amount was placed on moistened, precleaned microscope slides and dried by ignition of the fixative by passing the slide through a flame. The slides were then stained with Giemsa stain.

Results. In order to determine the optimum concentration of the mitogenic agent and the peak point of blastogenic response, the proliferative response of mouse lymphocytes to PHA was first investigated. Blood from 2–3 mice was pooled, the leukocytes were separated, and 1.5 $\times$ 10^6 nucleated cells were cultured in 1-ml volumes together with different concentrations of PHA, using two-fold dilutions of PHA-M. Triplicate cultures were pulsed daily for 16 hr with 1.0 μCi of ^{3}H-TdR and the response was measured (either by incorporation of the label to acid-insoluble fractions or by autoradiography and labeled nuclei counts). In another type of experiment, to ascertain the uniformity of the response both within and between different strains, cultures were originated from individual mice, established using an optimum dilution of PHA, and pulsed and harvested using similar schedules.

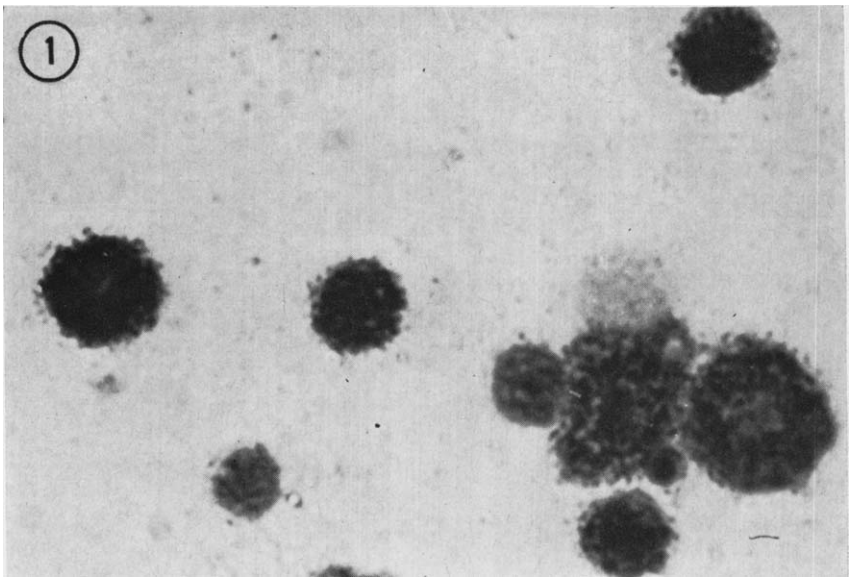

FIG. 1. Autoradiography of 3-day-old PHA-stimulated culture after 16-hr pulse labeling with ^{3}H-TdR. May-Greenwald-Giemsa; 1000×.

The best response was obtained with 1:150 (final) dilution of PHA-M (Table I) and the peak of the proliferative response took place at day 3 + 16 hr in culture. When different mouse strains were tested in similar kinds of experiments, the result was essentially the same. Individual animals also responded to PHA in a similar manner. Table II shows results of three separate experiments in which three individual (pertussis-treated) CBA mice were separately tested. The maximum response, again, took place at the third day in culture and no major differences between the responses of the lymphocytes of the three cell donors were observed. The autoradiographic data indicated that at the third day of culture 60–90% of the cells were undergoing DNA synthesis (Fig. 1).

TABLE I. Effect of Phytohemagglutinin[a] Concentration on the Stimulatory Effect of CBA Mouse Peripheral Leukocytes.

Pulse (hr in culture)	Net cpm/culture[b]		
	1:75	1:150	1:300
0 + 16	—	125	—
24 + 16	135	6810	5050
48 + 16	225	11050	5850
72 + 16	545	15000	6010
96 + 16	420	13200	4080

[a] PHA-M, Difco.
[b] Mean of triplicate cultures.

TABLE II. Stimulation of DNA Synthesis in Peripheral Leukocyte Cultures of Individual CBA Mice by Phytohemagglutinin[a] Uptake of ^{3}H-TdR.

Pulse (hr in culture)	Net cpm /culture[b]		
	Mouse no. 1	Mouse no. 2	Mouse no. 3
0 + 16	122	140	135
24 + 16	5500	8740	7090
48 + 16	9150	11220	11260
72 + 16	11040	17090	14160
96 + 16	12020	14195	13140

[a] PHA-M (Difco) diluted 1:150.
[b] Mean of triplicate cultures.

Cultures for metaphase analysis were set up in a similar manner; 1-1.5 × 10^6 nucleated cells were cultured in 1-ml volumes for 3 days and 0.1 μg of Colcemid was added to the cultures at approximately 70-72 hr of culture. The cells were harvested 2–5 hr later. When cultures were initiated from nontreated animals, usually one to two cultures could be established and there were approximately 100–300 metaphase plates/culture. Pretreatment with pertussis, however, sub-

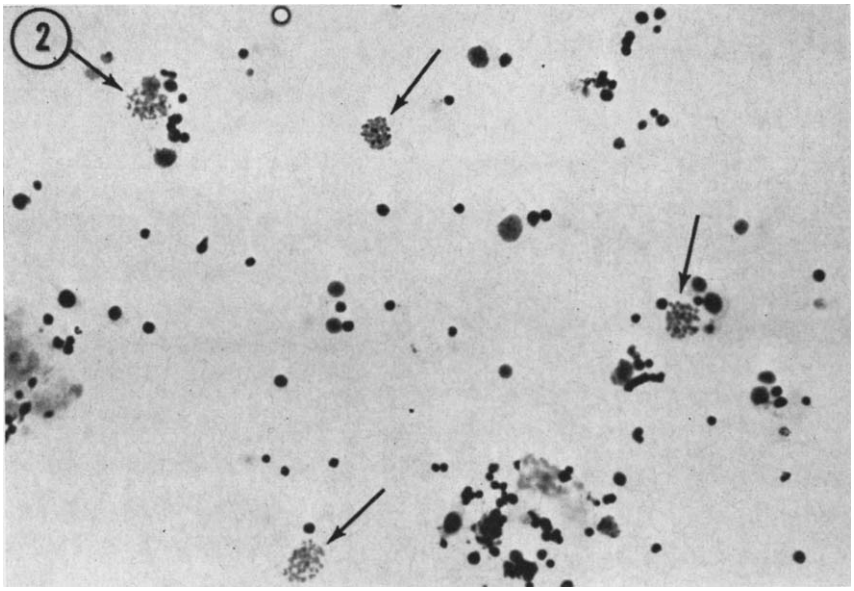

FIG. 2. Low-power photomicrograph of Colcemid-arrested culture at peak point of response. Arrows indicate metaphase plates; Giemsa; 76×.

stantially increased the number of cultures that could be established. From these pretreated animals, 5–15 cultures could be set up, and the yield of metaphase plates was accordingly 10 times higher. All of the mouse strains tested gave approximately similar results. Figure 2 shows the low-power microphotograph of a PHA-treated culture at the third day after 3 hr of Colcemid treatment, and in Fig. 3, two metaphase plates are shown.

Discussion. In most previous attempts to obtain chromosome preparations from mouse peripheral blood, the main difficulty was obviously the rapid loss of lymphocyte viability in culture at a time when the peak of response to the mitogenic agent had not yet been reached. By applying the separation

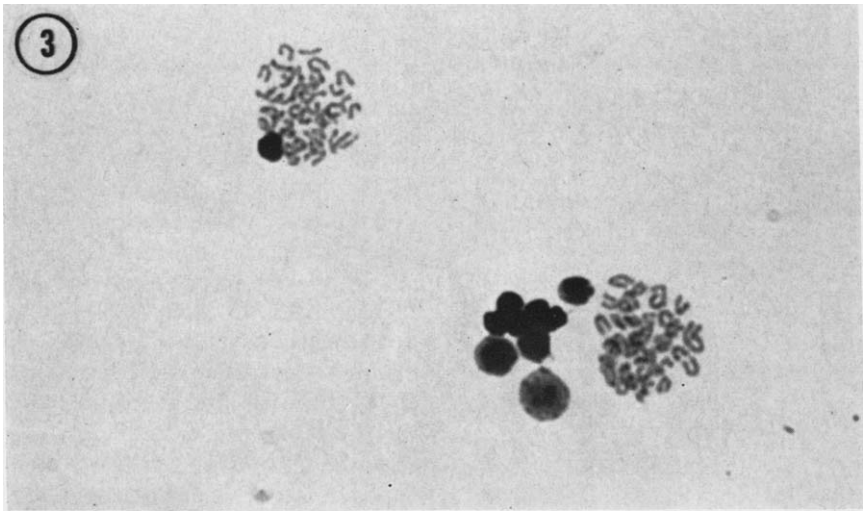

FIG. 3. Two spread metaphase plates; Giemsa; 1200×.

methods of Tridente *et al.* (9) and some of the culture conditions of Festenstein (10), satisfactory blastogenic responses to PHA and a large number of well-spread metaphase plates can be obtained.

The method of leukocyte separation and the type and the amount of serum used were of crucial importance for the cultivation of mouse peripheral leukocytes. Calf serum and animal sera from other sources (chicken, horse, rabbit) were inferior to fetal calf serum. Separation methods leaving more than 25-50 "contaminating" erythrocytes/leukocyte tended to suppress or inhibit the reaction (12). It was necessary to wash the separated leukocyte suspensions well, because either mouse plasma or residual amounts of plasma gel tended to suppress the reaction.

The peak response to PHA invariably took place at the third day in culture. Using the precautions for the culture conditions described, the viability of the peripheral blood cultures was 50–70% (measured using trypan blue uptake and compared to original inoculum).

Relatively little is known about the mechanism of the leukocytosis caused by pertussis organisms or by pertussis supernatant fluid administration. This phenomenon has been described and extensively studied by Morse and Bray (11). Cell yield can be considerably enriched by pretreatment of the animals with the *B. pertussis* suspension or pertussis culture supernatant fluid, as indicated by Morse. Therefore, the combination of these various techniques offers a reliable method for repeated sampling and analysis of the cytogenetic composition of mouse peripheral lymphocytes.

Summary. A method for chromosome preparations from mouse peripheral lymphocytes is described. This method is based on the mobilization of lymphocytes from lymphoid tissues to peripheral blood by pertussis vaccine or by supernatant fluid from *B. pertussis* culture, on the effective separation of leukocytes from the erythrocytes by sedimentation with Plasmagel® and on the use of phytohemagglutinin (PHA) as mitogenic agent. Large numbers of well-spread metaphase plates suitable for detailed chromosome analysis can be obtained from a single mouse without sacrificing the animal. The procedure can be applied repeatedly and it was successful with all strains of mice tested.

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