Tissue Culture of Rat Leukocytes. (29609)

ARCHIE A. MAC KINNEY, JR. AND WILLIAM L. KOPP (Introduced by R. F. Schilling)
Veterans Administration Hospital and Department of Medicine, University of Wisconsin Medical School, Madison

Short-term cultures of rat peripheral blood leukocytes have usually failed to proliferate, as 2 published reports(1,2) and several personal communications have attested. We have evolved a method in which the rate of cellular division is comparable to that obtained in cultures of human leukocytes. This paper describes the method, the variation of mitotic indices of rat leukocyte cultures, and also the effect of inducing delayed hypersensitivity to phytohemagglutinin.

Method. Leukocyte culture. Female Sprague-Dawley rats, 150 to 250 g, were anesthetized with intraperitoneal injections of 8 mg sodium pentobarbital. The skin was shaved and scrubbed with organic iodide* and 70% ethanol. Five to six ml of cardiac blood were aspirated and transferred to sterile 100-ml bottles. Blood was defibrinated by swirling in 120 ml glass bottles without glass beads. Blood from 3 animals was pooled. One ml of partially purified phytohemagglutinin protein 1:10 (PHA-P, Difco Laboratories, lot 457925) was added to each 20 ml of blood to aid sedimentation of erythrocytes. The mixture was allowed to stand 30 to 60 minutes at 40°C and was centrifuged 5 minutes at 400 rpm ($35 \times g$). Five ml of leukocyte-rich serum were removed from each bottle. Cultures were prepared with 2 ml leukocyte-rich serum, 8 ml NCTC 109,† and 0.03 ml PHA-P 1:10. Glassware was cleaned with sodium metasilicate. Cultures for bacterial contamination were made in thioglycollate broth.

After 3 days' incubation at 37°C leukocytes were exposed to 10^{-7} M colchicine for 2 hours. Air-dried metaphase preparations were made. Two workers counted the mitoses in 1000 cells. No significant difference between the duplicate counts was found. Mitotic indices were expressed as per cent mitoses per hour's exposure to colchicine.

Induction of delayed hypersensitivity. Seventy-two rats were randomly distributed into 3 groups. One group was injected in a hind foot pad with phytohemagglutinin mucoprotein (PHA-M, Difco Laboratories, lot 461977, 4.6 mg protein per ml by Kjeldahl analysis). Each animal received 0.1 ml of an emulsion which contained equal parts of PHA-M and Freund's adjuvant with 4 mg killed *M. tuberculosis* per ml. The second group received 0.05 ml of adjuvant without PHA-M. The third group received no injection.

To test for the presence of delayed hypersensitivity, 0.1 ml of PHA-M 1:10 (46 μg) in buffered saline was injected intradermally on the shaved abdomen. Criteria for delayed hypersensitivity were: 1) Induration of at least one centimeter in diameter, which was absent at 4 hours and maximal at 24 to 48 hours; and, 2) Characteristic histologic changes, which included mononuclear infiltration in the lower dermis and subcutaneous tissues(3). Fifteen of eighteen rats immunized in this manner with PHA-M showed typical delayed skin reactions when tested 5 days after the sensitizing dose. Other conditions failed to give positive reactions. Four animals tested at intervals of 10 or more days after immunization showed negative skin tests. Twelve rats injected with an emulsion in which antigen was diluted 1:10 or 1:100 showed negative responses at 5 days. The immunized groups were bled for leukocyte cultures 6 days after sensitization.

The experiment was performed twice with the same batch of material. The data from both experiments were pooled for analysis.

* Wescodyne: West Chemical Products, Inc., Long Island City, N. Y.

† NCTC 109: Difco dry medium made up to 1 liter with deionized water; pH brought to 7.1 with 10% sodium bicarbonate at 37°C; filtered under pressure with Millipore filter 0.22 μ. Streptomycin 120 mg/l, penicillin 60 mg/l were added to each 100 ml at time of use.

TABLE I. Tissue Culture of Rat Leukocytes from Normal and Sensitized Animals.

	No. of cultures	Mitotic index, %	Range, %
Control	16	1.0 ± .8*	.2–2.4
PHA + adjuvant	16	.5 ± .7	.0–2.5
Adjuvant	16	.4 ± .4	.0–1.6

* Mean ± S.D.

Results. Table I shows the analyzed data. Cultures from normal rats had a mean mitotic index of 1.0% per hour's exposure to colchicine. Both treated groups had significantly lower mitotic indices. The means of these two groups, however, were not significantly different. The range in each group demonstrates the wide variability in cultures of rat leukocytes.

Injection of adjuvant caused marked swelling of the injected extremity and regional lymph nodes but no other gross abnormality.

Discussion. Tissue culture of rat leukocytes has not been widely used, probably because a good method has not been available. Nichols and Levan(4) made chromosome preparations from cultures of rat leukocytes but did not report the consistency of the result or the degree of proliferation. Trowell (5) maintained rat lymphocytes in culture without PHA, but with low mitotic indices. Galton and Holt(6) reported mitotic indices of 1% in some cultures of leukocytes from another rodent, the golden hamster, when they used PHA. Since this work was completed, Dowd and coworkers(7) have reported that adequate metaphase preparations were obtained in 70% of rat leukocyte cultures. Their method is similar to ours except that glutamine and newborn calf serum were used in their preparations. They also reported that folic acid enhanced mitotic stimulating activity of PHA.

This method for rat leukocyte culture is a modification of the common method for human leukocyte cultures. Defibrination by swirling was used since heparin did not prevent clotting when the recommended concentration of 0.2 mg/ml was exceeded(8). Pooling of blood is not necessary if small volumes of culture material suffice. Various combinations of enrichments including 20% horse serum, 20% fetal bovine serum, 30 mg glutamine/100 ml, and 1 mg folic acid/100 ml did not consistently increase proliferation of cultures. The study of enrichments was hindered by failure to recognize the wide variation of mitotic indices in normal cultures. Each modification of the method required many animals to assess its effect on the mitotic index. Consequently, we cannot justify each step in the procedure. This method is simple and gives mitotic indices comparable to cultures of human leukocytes.

The methods for rat and human cultures employ PHA as a mitotic stimulant. Phytohemagglutinin was first used as a hemagglutinin to separate leukocytes from erythrocytes for tissue culture(9). Nowell(10) demonstrated that PHA was a mitotic stimulant of leukocytes. MacKinney and coworkers (11) showed that leukocytes which respond to PHA are principally small- and medium-sized lymphocytes.

Other agents have been demonstrated to stimulate mitosis or "blast" formation in human leukocytes *in vitro*. These agents include tuberculin(12), staphylococcal filtrate (13), homologous leukocytes(14), and tetanus and diphtheria toxoids(15). The most thoroughly studied of these agents is tuberculin, which stimulates proliferation of cultured leukocytes from healthy subjects with delayed hypersensitivity to tuberculin(16). The proliferative response, however, is slower and less intense than that induced by PHA. Whether tuberculin or other agents act in the same manner as PHA is uncertain.

If stimulation of leukocytic proliferation *in vitro* requires delayed hypersensitivity of the donor, induction of delayed hypersensitivity to PHA should enhance mitotic activity in cultures which contain this material. In this experiment, induction of Jones-Mote delayed hypersensitivity(17) did not increase the mitotic index. On the contrary, cultures from animals which had received an injection of adjuvant or emulsion showed significantly lower mitotic indices than cultures from normal rats. The inflammation in the injected extremity may have nonspecifically depressed mitotic activity in these groups. The stimu-

lation of cell division by PHA does not appear to require induction of delayed hypersensitivity in the rat.

Summary. A method of culturing rat leukocytes with phytohemagglutinin has been described. The variation of mitotic indices of 16 cultures from 48 normal Sprague-Dawley rats has been shown. The mean mitotic index was 1%, which is comparable to values for normal cultures of human leukocytes. Induction of delayed hypersensitivity to PHA did not enhance proliferation in this species.

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Serum Phosphocreatine Kinase in Hereditary Muscular Dystrophy and Cardiac Necrosis of Syrian Golden Hamsters.* (29610)

MONIKA EPPENBERGER, C. W. NIXON, J. R. BAKER AND F. HOMBURGER
Bio-Research Institute, Cambridge, Mass.

Markedly increased serum phosphocreatine kinase activity has been reported by Aebi *et al*(1) and Dreyfus and Schapira(2) in the Duchenne type of muscular dystrophy in children even before the onset of clinical manifestations, and moderate increases in serum enzyme level prevail in heterozygote carriers of the gene which transmits this disease in man(3,4,5). In this paper are reported elevated serum phosphocreatine kinase levels in Syrian hamsters with hereditary muscular dystrophy and cardiac necrosis(6), and changes of serum enzyme levels which occur during the progress of the dystrophy and cardiopathy.

Materials and methods. Sixty dystrophic (BIO 1.50 and BIO 14.6) male and female hamsters divided among 6 age groups, 10 heterozygote F_1 hamsters from a cross between BIO 1.50 and other (non-dystrophic) strains, 50 non-dystrophic hamsters of both sexes and similarly divided as to age, were studied. Ages of the controls ranged from 13 to 458 days and those of the dystrophic animals, from 13 to 230 days. The hybrids were from 129 to 220 days old. The presence or absence of dystrophy was determined by histologic examination of the heart and 6 different muscles of each animal. The histological criteria for diagnosis of dystrophy were presence of rowing nuclei, inequality of fiber

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