REDUCED LEUKOCYTE VOLUME TECHNIQUE FOR HUMAN CYTOGENETIC ANALYSIS[1]

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Introduction

Recent progress in human cytogenetics is mainly due to the improvement of technique. In 1956, Tjio and Levan (1956) discovered that the number of human chromosomes in tissue culture cells was 46 rather than 48. The result was soon confirmed by Ford and Hamerton (1956). Subsequent studies on human chromosomes were made by using the tissue culture technique and employing a variety of tissues: skin, muscles, and bone marrow (Ford, 1962).

More recently, the method of short-term culture of leukocytes from peripheral blood has been introduced as a reliable approach to chromosome studies. A standard tissue culture basal medium, TC 199, has come into general use and the method as improved by Moorhead, *et al.* (1960) has now been generally adopted (Miller, *et al.*, 1962; German, *et al.*, 1962). The greatest advantages of this method are: (1) A few mililiters of blood are sufficient to initiate a culture. (2) Dividing cells are obtained in three days and very limited precaution in asepsis is required. (3) There is no possibility of a clone with a variant karyotype arising and becoming an important component of the population of cells during the short period of culture. Since the sample of blood can be presumed to be derived from all hemopoietic sites, the karyotype observed should therefore be more truly representative of the whole body than the karyotypes derived from methods employing tissue biopsy.

The standard Moorhead method (Moorhead, *et al.*, 1960) of blood culture gives excellent preparations for cytological study, but is inefficient in the sense that some 3×10^7 lymphocytes, or $7\text{–}8 \times 10^7$ leukocytes (Nonidez and Windle, 1953) are obtained by venipuncture and withdrawal of 6–10 ml. of blood. The yield of mitotic plates far exceeds the minimum necessary for reasonable cytological interpretation, and as the method is applied to the study of infants and

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children it is not only wasteful but imposes several restriction and complications. Development of a simple and reliable method with reduced blood volume requirement would be advantageous in familial and small scale population studies. Blood withdrawal by lancet puncture would be more convenient for field work and group study; in addition it could be done by a geneticist independently of a certificated medical technician.

It was the intent of this study to compare the existing techniques for leukocyte culture (Moorhead, *et al.*, 1960; Edwards, 1962) and to develop innovations that would permit further reduction in total blood volume, without loss of cytogenetic validity.

Review of Literature

In 1939, Kemp, the Danish human geneticist, attempted the study of chromosomes in tissue culture in order to avoid the distortion arising in microtome sections (Levan, 1960), but in processing his material according to the available technique of the time, he failed to obtain plates suitable for critical analysis.

Since the advent of modern tissue culture technique (Tjio and Levan, 1956) many tissues have been cultured for human chromosome study. Human bone marrow *in vitro* was first used by Laitha (1952). The first general method developed for using small skin biopsies as a source of living cells was that of Tjio and Puck (1958). Other methods have been introduced by Chu and Giles (1959), Edwards (1960), Harnden (1960), Hayflick and Moorhead (1961), Ingenito, *et al.* (1958), Nilsson, *et al.* (1959), and Nowell (1960a).

The most recent development, which shows great promise, is the short-term culture of cells from blood. Chrustchoff (1935) first did cytological investigations on cultures of normal human blood. In 1955, a technique called the gradient method was developed (Osgood and Brooke, 1955; Osgood and Krippaehne, 1955); in which leukocytes from peripheral blood were grown *in vitro*. The method was first used for chromosome studies by Hungerford, *et al.* (1959). The details have now been modified and results improved by Moorhead, *et al.* (1960) and Arakaki (1961). In order to karyotype aborted fetal material, neonates and infants reduction of blood volume (Edwards and Young, 1961; Froland, 1962) is important. A simple method for making chromosome preparations from a very small volume is described by Edwards (1962). This method employs blood from a skin puncture (or a skin biopsy) (Edwards, 1960), a procedure not requiring any medical supervision. Most recently Arakaki and Sparkes (1963), independently of this study, described a method for substituting whole blood for plasma-suspended leukocytes in the leukocyte culture technique.

The fundamental requirements of a good preparation are sufficient induction of cell division, reliable fixation and non-overlapping dispersion of contrastably stained chromosomes. Phytohemagglutinin (PHA), an extract of *Phaseolus vulgaris* (red kidney bean), first used as a red cell agglutinin in the separation of white cells (Skoog and Beck, 1956), has since been shown to stimulate mitotic activity in white cells as well (Hungerford, *et al.*, 1959; and Nowell, 1960b). It is now in standard use as a mitogenic substance in short-term leukocyte cultures (Moorhead, *et al.*, 1960; and Nowell, 1960a).

Chemical analysis of the original PHA extract showed it to be protein in character (Goddard and Mendle, 1929) but later a mucopolysaccharide group was identified by electrophoresis (Rigas and Osgood, 1955). The agglutinating property of the substance was found to be the protein fraction, but the agglutinating and mitogenic factors are evidently not the same (Beckman, *et al.* 1962).

The mechanism for the effect of PHA on leukocyte mitosis has been discussed in several papers (Nowell, 1960b; MacKinney, *et al.*, 1962; Elves and Wilkinson, 1963). Apparently PHA promotes both a degeneration of polymorphonuclear cells and a conversion of lymphocytes to primitive blast-like cells. It is the latter cells, similar to the blast cells of bone marrow, that divide mitotically. Although various hypotheses have been proposed as to the physiological initiation of this conversion of lymphocytes, including release of trephones from degenerating lymphocytes, no convincing mechanism exists for any of them.

The basal synthetic medium, TC 199, containing various amino acids, vitamins, nucleic acid constituents and various accessory growth factor was devised by Morgan, *et al*, (1950). Despite its complexity, TC 199 is unable to maintain cells for more than one or two days unless serum is added. Morgan has subsequently produced a modified TC 199 essentially by substituting Hank's Balanced Salt Solution (BSS) for Earle's BSS (Paul, 1960).

The period of short-term leukocyte culture is 2–5 days at 37° C (body temperature). Usually the cells are harvested after three days of incubation (65–70 hr. Moorhead, *et al.*, 1960). It has been found that DNA synthesis begins at 24 hours and by 72 hours, 40–45 per cent of cells are synthesizing DNA (MacKinney, *et al.*, 1962). Edwards (1962) found that mitosis was maximal at about 48 hours, and was sometimes followed by a fallow period of up to 12 hours. Early harvesting may coincide with this temporary depression in mitotic activity and harvesting too late may result in missing most of the mitotic cells.

Further improvements in cytological technique have contributed to human cytogenetic applications. Colchicine and other spindle fiber inhibitors such as colcemid and vincaleukoblastine have been important as means of arresting mitoses at metaphase, causing accumulations of metaphase figures, and increas-

ing chromosome contraction (Ford, 1962; Ford and Hamerton, 1956; Palmer, *et al.*, 1960; Rendon, *et al.*, 1962; Tjio and Levan, 1956). It has been proven possible to maintain the cells in this colchicine induced strongly hydrated state and yet achieve satisfactory fixation and staining.

Hsu (1952) discovered that exposure of tissue culture to hypotonic solution not only causes the cell to swell but through osmotic dissolution of the spindle as well, the chromosomes do not congress at metaphase but remain dispersed throughout the cytoplasm (Hungerford and Dibernardino, 1958). Sodium citrate was used in Hsu's experiment and later a one-fourth strength dilution of Hank's balanced salt solution was used instead (Moorhead *et al.*, 1960). Lejeune, *et al.*, 1959, suggested hypotonic serum be used to induce spreading; Hungerford (1962) has shown this to be a gainful substitute for a diluted salt solution.

For the preparation of slides, the squash method originally introduced by Heitz (1936) for plant material was first used. As an improvement upon mechanical squashing, the rapid air-drying of cells on slides after fixation was introduced (Rothfels and Siminovitch, 1948; Tjio and Puck, 1958); some workers even ignited the fixative to achieve instant drying as in Dr. Miller's laboratory.

For the staining procedure, aceto-carmine, aceto-orcein, Wright's stain, Feulgen reagent and Giemsa have been used (Miller, *et al.*, 1962; Moorhead, *et al.*, 1960; and Nilsson, *et al.*, 1959).

For karyotype analysis the classical method of photographic enlargement followed by idiograph construction has been used (Rayleigh, 1891). A standard system of nomenclature for human mitotic chromosome was proposed and developed by the human chromosome study group (Böök, *et al.*, 1960), but it is variously accepted by investigators in the field.

Material and Methods

In this study, Moorhead's (1960) and Edwards' (1962) methods have been repeated and the results are compared. A method of whole blood culture has been devised. The elements common to all experiments are given immediately below, followed by brief descriptions of Moorhead's and Edwards' methods, and a complete description of a new method.

Preparation of Medium: TC 199 (Cappel), with Hank's balanced salt solution as base was used for all the cultures in this experiment. The antibiotics, penicillin (crysticillin 300 A.S., Squibb procaine penicillin 6 in aqueous suspension, 300,000 units per ml.) and streptomycin (Parke) were added to the media to achieve a final concentration of 200 unis/ml of penicillin and 200 μg/ml of streptomycin.

The 10X stock medium was diluted with sterile distilled water and 15–20

per cent of patient's plasma or human serum (Difco) was added. Adjustment of pH to approximately 7.3–7.4 was made with 0.1N HCl or 10% Na_2CO_3.

Difco Bacto-phytohemagglutinin P-form in hemagglutination buffer was added at a concentration of 0.01 ml/10 ml of medium. Heparin in an aqueous ammonia solution was used to prevent coagulation.

Culture of Leukocytes: Cells were cultured in upright tubes or horizontally placed bottles for 72 hours at 37° C. Four to five hours before harvesting either 0.1% colchicine (Turtox) was added at a proportion of 0.2 ml/10 ml medium or 0.04% colcemid (CIBA) at a proportion of 0.5 ml/10 ml medium.

Harvest and Fixation of Cells: Cell suspensions were centrifuged and the supernatant fluid removed by a Pasteur pipette. Cells were then resuspended in hypotonic solution either 1/4 strength of Hank's BSS (Difco) or 1/6 strength serum (diluted with distilled water) and incubated for 10–25 min. Again the cells were centrifuged and the supernatant discarded. Freshly made fixative (1:3 glacial acetic acid and absolute methyl alcohol) was introduced without disturbing the "button" of cells. After 30 minutes in the fixative, button of cells was disrupted, centrifuged, and the supernatant fluid replaced by fresh fixative. The top fine layer of leukocytes was then transferred to a small test tube (10× 75 mm.).

Cytological Preparation: To prepare the cells for cytological examination, two drops of cells suspension were placed on a chilled slide; blowing on the drops abetted the required spreading. The slide was then rapidly dried by passing it over a small flame, allowing the slide temperature to only slightly exceed body temperature.

Staining was done with the common Wright certified blood stain, made as follows:

Wright's staining Solution

Wright's stain.....0.1 gm.
Acetone free absolute methyl alcohol.....60.0 ml.
Agitate daily for 5 min. for more than 15 days,
then filter and use.

Wright's buffer solution

Potassium phosphate (KH_2PO_4).....3.165 gm.
Sodium phosphate dibasic (Na_2HPO_4).....1.6 gm.

Distilled water, q. a.....500 ml.

Before staining, the acetic acid was removed by rinsing the slides in two changes of acetone-free absolute methyl alcohol. Slides were then flooded with a 1:1 mixture of Wright's stain and buffer. This resulted in the appearance of a greenish metallic scum. The optimum staining time varied with the volume of stain used, 2–3 minutes usually gave good results. Then the stain was flushed off with distilled water and rinsed till every trace of the stain solution was lost. Slides were either left like this or mounted in diaphane as soon as dry.

Moorhead Method, (as modified by Nowell, 1960a, and Arakaki, 1961): The essential feature of this method is the culture of leukocytes separated from about 10 ml. of venous blood. PHA is used both in the separation of white cells initially and as a component of the culture medium to induce mitosis. Since the detail of the method is described adequately elsewhere (Arakaki 1961), no further description is given here.

Edwards Method, as modified: This technique is unique in that it begins with 0.5–1 ml. of venous blood and that initial separation of white cells is accomplished by centrifugation within the original hypodermic syringe. By bending the needle, white cells are successfully discharged into a culture flask, and thereafter the method is essentially that of Moorhead. Detail is described by Edwards (1962) and was followed here except that an ordinary air incubator environment was substituted for 5% CO_2.

New Micro-Method: In devising a new micro-method with reduced blood volume requirement, various modifications and innovations were attempted. These included variations in time of harvest, proportion of white cells to medium, and variations of culture vessel volume. The method described represents the combination of variables which appeared to give best results.

Three to ten drops (about 0.1-0.3 ml.) of capillary blood were collected from a finger prick with a sterile, disposable lancet (Becton Dickinson & Co.) and placed directly into a heparinized, sterile test tube (10 × 75 mm.). Two ml. of the following solution were added.

Medium TC 199 (Cappel)..... 80%
Human or Calf serum..... 20%
Streptomycin.....200μg/ml
Penicillin.....200 units/ml
with or without 1/10 PHA-P.....0.1 ml/5ml of medium

If the medium contained no phytohemagglutinin, two drops of 1/10 PHA-P

were added. Test tubes were plugged with sterilized corks or cotton and then incubated at 37° C for three days (72 hr.). Four hours before the cells were harvested, 0.1ml. (3 or 4 drops from a 20 gauge needle) of 0.04% colcemid or colchicine was added, and the tube was shaken. At the end of 4 hrs. the cells in the test tube were suspended and centrifuged at 800 rpm for 5 min. The supernatant fluid was pipetted out, and the cells were resuspended in 1.5 ml. of 1/4 strength Hank's solution or a 1/6 concentration of hypotonic serum. Tubes were then incubated at 37° C for 10-15 min., (25 min, if hypotonic serum was used) and then centrifuged at 600 rpm for eight minuites. The supernatant fluid was pipetted out by pasteur Pipette. One and one-half mililiters of fixative were added without disturbing the "button" of the cells. After the tubes stood at room temperature for 30 minutes, the button of cells was broken by aspiration with a pipette, and the cells centrifuged at 300 rpm for 10 min. The supernatant fluid was carefully pipetted out and fresh fixative was added. The fine layer of leukocytes on the top was transferred to another clean test tube and suspended in 0.4-0.6 ml. of fixative. Cytological preparation was identical to that mentioned in the former section. Up to 15 slides could be made from each culture depending on the initial volume of cells and circumstances of the preparation.

Results and Discussion

For a successful culture it is necessary to have a high percentage of mitotic cells with enough good figures so that karyotype analyses can be used to determine, with some reliability, the chromosome number. In a dependable analysis, at least 30 counts of the chromosomes are required since they will vary somewhat even in the normal individual. To study chromosomal structural aberrations, such as translocations and ring chromosomes, it is assumed that the preparation of at least 5 karyotype analyses will be reliable.

It was the intention in this project to satisfy the above criteria and, at the same time, simplify the culture method and, reduce the amount of starter blood. Some sixty cultures were affected in order to test several variables but mainly to examine the effects of reduced cell number. For twenty-four of the cultures, blood was collected in a syringe, in the remainder, directly in tubes, These cultures are from sample sources as indicated in Table 1.

Table 1. *Summary of sample sources*

Ethnic group		Chinese	Caucasian	Filipino	Egyptian
Sex	Female	2	5	1	0
	Male	5	1	0	1

For the whole blood micro-method, the percentage of mitotic cells varied from 0.45–3.34 % (Table 2.) and the average frequency of mitotic cells was 1.18 %. The limited data certainly suggest that the whole blood method is equal in recovery to the other two. Considering that 15 slides could be made from each culture, all the cultures, except 34–B, are easily successful according to the criteria previously mentioned.

Table 2. *Evaluation of mitotic success from different leukocyte culture methods*

Sources	Culture No.	Mitotic cells/slide			No. of total cells/slide	Percentage, Mitotic cells
		"Karyotypable"	Countable	Total		
Moorhead's method	6	—	—	48.2	5,290	1.46
	9	—	—	14.8	3,150	0.46
						$\bar{x}$=0.96
Whole blood culture collected by syringe	14	1	3	4	972	0.41
	23	4	12	26	1,023	2.54
	25—A	1	9	13	1,622	0.80
	27—B	1	4	7	1,104	0.63
	29—C_2	—	6	17	1,165	1.46
						$\bar{x}$=1.15
Whole blood culture directly collected in test tube	30—A	8	21	49	5,360	0.93
	33—A	—	—	36	4,526	0.80
	33—C	2	10	30	4,221	0.71
	34—A_1	4	8	12	1,510	0.79
	34—B_1	0	2	25	1,603	1.56
	34—C_1	1	5	23	2,249	1.02
	34—D_1	2	12	21	4,698	0.45
	34—E_1	1	5	11	818	1.34
	34—F_1	2	19	64	1,914	3.34
	34—G_1	6	13	43	5,040	0.87
						$\bar{x}$=1.18

The cultures beginning with 34 are from the final test for the whole blood culture method. Fourteen cultures were made with the blood from 6 individuals. Table 3 is a breakdown of further variables in this experiment. No quantitative differences effect of serum source or blood volume are given. However, certain subjective evaluations will be discussed.

Two samples were taken from a single prick. There was a general tendency for the first culture to be better than the second although there was often a larger amount of blood to start with. In the second sample total cell counts were usually decreased, and more degenerate polymorphonucleate cells were observed. Therefore if one wants to take more than one sample to make sure

Table 3. *Summary of treatments of final experiment*

Culture No.	20% serum from	Drops of blood
34—A_1, A_2	human	5, 7
34—B_1, B_2	calf	5, 7
34—C_1, C_2	calf	4, 8
34—D_1, D_2	human	4, 8
34—E_1, E_2	human	4, 8
34—F_1, F_2	calf	4, 8
34—G_1, G_2	human	4, 8

that the culture will be successful, better results might be obtained from separate pricks.

In testing the method, a blood sample from one to ten drops was used for each of the cultures. The experimental results show that no successful culture was obtained from initial blood sample less than three drops; in other words, at least 0.1 ml. blood is necessary for a successful culture.

Aseptic cleansing of the finger with 95% alcohol before the blood was sampled was necessary to prevent becterial contamination. With cleansing only four out of 36 whole blood cultures were contaminated by bacteria. Three of these were sampled from the same person, and the other was sampled at the same time, but from another individual. Undoubtedly, more careful technique would reduce contamination to essentially zero.

Small vials (12×50 mm.) with flat bottoms were sometimes used for culture instead of test tubes. The cells grew well, possibly better, in these vials because of the broader surface area, but it was more difficult to separate the fine layer of leukocytes from the flat bottom. The standard 10×75 mm. culture tubes appear generally satisfactory.

Calf serum and human serum were used in this study, and both were satisfactory. Human serum appeared to be better than the calf serum but only slightly so.

As far as colchicine and colcemid are concerned, both are satisfactory. Ideal concentrations appeared to be about 0.04%.

Hypotonic Hank's salt solution and hypotonic serum were used for prefixative treatment, and both were satisfactory. Only slight differences between the two were found.

Because of the small volume of medium and limited size of test tube, the acidity (pH value) was often found to change during the period of culture. As a result readjustment of the pH value was sometimes necessary. The most frequent change was for the medium to become acidic as shown by the phenol red indicator. For readjustment, the usual ten per cent Na_2CO_3 was too high a

concentration for the tiny volume of culture; 5 per cent Na_2CO_3 solution was used for this purpose instead.

After harvesting was completed, a slide was made to check for adequacy of the culture. If it was satisfactory, it was found best to prepare the slides as soon as possible. Delay of preparation impeded spreading of the cells as well as the chromosomes. In addition, delay of slide preparation impaired the staining ability of the chromosome and also resulted in a coloration of the cytoplasm.

The spreading of cells in the whole blood culture method was as good as in other methods. A brief description of the slide made from the last run of cultures is presented.

34-A—Mostly monocytes and lymphocytes, some with lightly stained cytoplasm. A small proportion polymorphonucleate cells and a few dinucleate cells were observed, and the cells were nicely spread out.

34-B—Most of the cells were well spred out, but a small proportion of them was clumped into small groups (3–10 cells, usually small lymphocyte or polymorphonucleate cells). Cells and chromosomes were both contracted.

34-C—Mainly lymphocytes, and some monocytes. Very few polymorphonucleate cells. Some of the cells were clumped, but spreading of leukocytes was otherwise good.

34-D—Many polymorphonucleate cells and small lymphocytes, the low proportion of mitotic cells was probably due to the low proportion of primitive blast-like cells.

34-E—Most of the cells were lymphocytes of all sizes followed next in number by monocytes. Cell density was low and the cells were well spread.

34-F—Relatively high numbers of cells were observed. Most of the cells were monocytes, followed by lymphocytes. Cells were quite well spread.

34-G—There were many cells on the slide. Cells were nicely spread. Occasionally 3–20 cells were clumped into a single group. Most of them were small lymphocytes followed next in number by large lymphocytes; monocytes and polymorphonucleat cells were less in proportion.

It has been reported that the small lymphocytes change to large, more primitive, cells which then divide (Elves and Wilkinson, 1963). In this study polymorphonucleate cells were seen to gradually disappear from the culture but some degenerate polymorphonucleate cells remained at termination of some of the cultures.

The following is a comparison of the results of Moorhead's and Edwards' methods with those of the whole blood culture technique used here. More cells than necessary are obtained with Moorhead's method and although good figures are easily obtained from such a culture, the method is wasteful. Moreover, a certified medical technician is required, and the method presents difficulties

with infants or fetal material. In Edwards' method, blood volume is reduced to between 0.5–1.0 ml. Preculture separation of leukocytes causes the loss of cells, especially with the small amount of blood used. Clumping of the cells is found, probably due to preculture centrifugation. The whole blood micro-method is very simple and less equipment is required. Ample figures are obtained from a low volume of blood and the method appears applicable to fetal material and infants as well as adults. Although it is not 100% successful, it can be easily repeated when occasional failures occur, and in any case, the likelihood of success is as good as with the other two methods.

In the experiment number 34, the cultures were left in the incubator for 24 hours prior to addition of PHA. Harvesting started 72 hours after PHA was added. The success of the culture suggests the possibility for delayed harvesting if the samples have to be sent to laboratories removed from the sites of collection.

Summary and Conclusion

A technique of whole blood culture, including harvest from the original container to avoid the loss of leukocytes in transfer, was devised. The minimum blood volume for a successful culture with this method is 0.1 ml., but 0.2–0.3 ml. of blood give a more dependable product than the minimum volume.

The whole blood culture method proved very practical and flexible. Less equipment was required and the simplicity makes it suitable for field work and group study. For this purposes, the mitogen, phytohemagglutinin, could be added to the medium at least 24 hours after collection. In cases where laboratory equipment is not available, and transportation over a long distance is necessary, the blood sample could probably be kept in the medium with serum for longer periods.

The reduced blood volume method permits cytogenetic analysis of fetal death, congenital defect of newborn, and follow-up of relatives on an out-patient basis.

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應用於人類細胞遺傳分析的少量白血球培養法

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以往應用在人類細胞遺傳研究的短期培養法是先把白血球分離出來，然後在培養瓶中培養白血球。這種方法須血 5～10ml. 但是不能應用於幼童。爲了減少用血量和操作過程中的損失以適於幼兒染色體的研究，本報告就是要介紹一種簡便的完整血培養法 (whole blood culture), 在原來取樣的試管中，培養並收獲白血球，用這一方法能得到成功培養的最低用血量，是 0.1ml.。但用 0.2～0.3 ml. 血可產生的結果是更爲可靠。

這種完整血的培養法，極爲實用，並且具有伸縮性，須要的設備少，而且方法也簡化，可適用於研究室以外的採樣工作和從事於大量採樣以作羣體的研究，當實驗室設備離取樣的地方相當遠的時候，可延遲加誘細胞分裂劑 (mitogen) phytohemagglutinin 的時間。這時可將血樣保存於含有血清的培養液裏，這樣可搬運到較遠的距離。

這一個少量血的培養法，可以分析胎兒死亡，新生兒先天性缺陷，並跟着分析他們親戚的染色體來從事於人類細胞遺傳的研究。

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