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Impact of lymphocyte culture media on the number of metaphases and chromosome band resolution*

Wpływ podłoży do hodowli limfocytów na liczbę metafaz oraz rozdzielczość prążkową chromosomów

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

A special type of differential staining of chromosomes is replication banding. This staining technique reveals the band pattern characteristic of each homologous pair of chromosomes, which is a reflection of heterogeneous euchromatin structure. Banding enables identification of homologous chromosomes and detection of chromosomal aberrations, both structural and numerical. Slide preparation requires knowledge of many techniques, and the procedure is often different for each laboratory. The aim of the study was to determine the effect of selected media for lymphocyte cultures on the number of metaphases and the band resolution of chromosomes. The study was carried out using cell cultures from whole peripheral blood. The slides were stained by the GTG method. After their removal from the water bath they were immersed in trypsin solution, then rinsed in PBS solution and stained in Giemsa solution. After staining they were rinsed again and left to dry. The study confirmed the effect of selected commercially available cell media on the number of metaphases and band resolution of chromosomes, which have not previously been described. In all of the tests performed, the cell culture, fixation, slide preparation (automatic method), staining, and number of reagents were identical.

Key words: **karyotype • chromosomes • cell culture medium • chromosome band resolution • karyotype analysis**

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Abbreviations: : **DME** – Dulbecco's modified Eagle medium; **FBS** – foetal bovine serum; **GTG** – trypsin and Giemsa chromosome staining technique; **KCl** – potassium chloride; **MEM** – modified Eagle medium; **Pb-MAX** – a complete medium; **PBS** – phosphate-buffered saline; **PHA** – phytohaemagglutinin; **PWM** – pokeweed mitogen; **RPMI** 1640 – a complete medium (Roswell Park Memorial Institute).

Introduction

The most complete information on the karyotype of a species is obtained by analysing mitotic chromosomes. Precise analysis of chromosomes, enabling identification of homologous pairs or aberrations, requires the use of differential staining – replication banding of chromosomes [2,15,17].

The main staining technique used to determine the band pattern of organisms included in karyotype standardization projects is GTG staining. The staining results in an arrangement of dark and light bands visible on the chromatids. The band pattern is a reflection of the heterogeneous structure of euchromatin. Positive, dark G-bands correspond to sites enriched with A-T pairs. They occur most often in non-coding sequences of the genome and contain far fewer genes than, for example, R-bands [2,4,6].

The greater the resolution of the sample – the number of bands in a given chromosome – the smaller the anomalies that can be detected in chromosome structure. Despite the apparent simplicity of individual procedures such as initiation and inhibition of cell cultures, obtaining high-quality cytogenetic smears is not a routine matter [3,11,13]. Factors influencing the result include the quantity of growth stimulants such as phytohaemagglutinin (PHA), the percentage content of serum (FBS) in the medium, the amount of antibiotic, the amino acid content of the medium, the density of the cell culture, the number of metaphase cells, the amount of colcemid (added to cells during active growth to keep them in metaphase), fixative, potassium chloride (KCl), the duration of the cell culture and of incubation in KCl, and the means of slide preparation [1,5].

Cell media

A culture medium should contain all substances necessary for stimulation of cell division and growth. The basic medium is usually RPMI 1640, containing HEPES and L- glutamine. A liquid medium should be enriched with FBS (foetal bovine serum), mitogenic substances such as PHA (phytohaemagglutinin) or PWM (pokeweed mitogen), and antibiotics protecting the culture against bacterial and fungal contamination. Mitogen stimulates T-cells to divide as early as the first 24 hours of culture, which is accompanied by biochemical changes. The RNA and DNA synthesis index increases sharply in the second 24 hours [12]. In some cases substances synchronizing cell division – methotrexate or thymidine – or preventing chromosome condensation – ethidium bromide, actinomycin D, BrdU or 5-azacytidine – can be added to the culture [8]. In mitotically active cells the most commonly used mitogen is PHA, a mucoprotein which occurs naturally in kidney beans (*Phaseolus vulgaris*). It is the most effective in stimulating T-cells by binding to the T-cell receptor complex [12]. Other media used for lymphocyte cultures include Eagle's MEM, DME, RPMI 1603, Medium 199, Ham's F-10 and McCoy's 5A.

Slide preparation

Factors influencing the result include the density of the cell culture, the number of metaphase cells, the amount of colcemid (added to cells during active growth to keep them in metaphase), the quantity of growth stimulants such as phytohaemagglutinin (PHA), the percentage content of serum (FBS) in the medium, the amount of antibiotic, fixative and KCl, the duration of the cell culture and the duration of incubation in KCl [5,10].

The next stage affecting the quality of the final effect of karyotype determination is the slide preparation method, developed in the form of protocols by independent laboratories. Significant variables forming the framework of the protocols include temperature, drying time, fixation, relative humidity and preparation of the microscope slide [13]. It is important to consider the height, width and thickness of the slide. Parameters such as the height from which the suspension is dropped on the slide are also significant [14]. In many methods for drying smears, after the suspension is dropped on the slide, the air-dried smear or smear left at room temperature is frequently used. In other methods, the smears can be dried in heated chambers or water baths with appropriate humidity. These different individual techniques make it difficult to obtain a result of high quality [10]. In order to increase the reliability and repeatability of the smears, special equipment for obtaining cytogenetic smears is increasingly used in laboratories. The use of automatic methods makes it possible to easily set parameters and create a uniform procedure for all samples, which has a significant effect on the final result. A research hypothesis was put forth that the quality of a cytogenetic smear (number of metaphases and band resolution of chromosomes) can be influenced by the type of cell medium and the duration of digestion in trypsin and of staining in Giemsa stain.

Preparation of smears suitable for further analysis remains a subject of great interest. Despite the apparent simplicity of individual procedures, obtaining high-quality cytogenetic smears is not routine. There are many factors influencing the result of the karyotype analysis.

The aim of the study was to determine the effect of selected media for lymphocyte cultures on the number of metaphases and the band resolution of chromosomes.

Materials and methods

Human tissue

The study was carried out using cell cultures from whole peripheral blood from 8 healthy patients – men and women aged 20-50 – collected into sodium heparin tubes. The material was collected with the consent of the director of the clinic with the patients' consent, according to the Bioethics Commission protocol.

Cell cultures and fixation

Cells from each of the patients were grown in 72-hour cultures on four different media: LymphoGrow (Cytogen, Princeton, NJ USA), Panserin 401 (PAN-Biotech GmbH, Aidenbach, Germany), PB-MAX Karyotyping Medium (Thermo Fisher Scientific, Waltham, MA USA), and our own medium consisting of RPMI (38.2 ml/50 ml medium; PAA, Laboratories GmbH, Cölbe, Germany), hereafter referred to as RPMI plus, phytohaemagglutinin (1.2 ml/50 ml of medium; Biological Industries, Israel), foetal bovine serum (10 ml/50 ml of medium; Biomed-Lublin, Lublin, Poland) and the antibiotics penicillin and streptomycin (0.6 ml/50 ml of medium; Thermo Fisher Scientific, Waltham, *MA USA*). Each cell culture contained 9 ml of medium and 0.8 ml of blood. Seventy-two hours after the start of the culture 110 µl of colcemid (Biological Industries Beit-Haemek Israel Ltd., Kibutz Beit-Haemek, Israel) was added in order to keep the cells in metaphase, after which the culture was incubated again at 37°C and in 5% CO_2 for 60 min. After this time the cells were treated with 10 ml of 0.075 M KCl hypotonic solution (Thermo Fisher Scientific, *Waltham, MA USA*) for 30 min at 37°C. The next step was to collect the cells by centrifugation and fix them by adding cold methanol and acetic acid (3:1) fixative. This was done three times. In the final stage, fixative was poured on the suspension and it was left in a freezer at -10°C.

Slide preparation

After the 32 cell cultures had been set up (four per patient), a total of 64 slides were prepared (8 per patient, two from each fixed cell culture). The smears were prepared by an automatic method using a Zendropper apparatus (Zentech S.A., Angleur, Belgium). Prior to use, the hydraulic system and needles were washed with a chlorine solution, as recommended by the manufacturer, and then they were rinsed in washing buffer and distilled water. After that the hydraulic system and needles were rinsed in washing buffer and distilled water. Then the needle was calibrated by placing physiological saline and fixative (3:1 methanol and acetic acid) at two marked positions.

The slides were prepared using the option of dropping the suspension on a slide previously moistened with fixative (wet slides). Owing to the program's advanced functions, it was possible to choose the size of the test tubes (15 ml) and to set the height of the needle in four positions with respect to the plate with the smears, the position of the needle with respect to the slide, the height of the stream of water dropped onto the slide, the height of the stream of sample dropped onto the slide, the volume of suspension placed on the slide, the number of repetitions per patient, and the time from the moment the fixative is dropped onto the slide to the moment the sample is placed on the slide. The prepared slides were placed in an incubator at 50°C for 48 hours.

Staining the slides

The slides were stained by the GTG method. After they were removed from the water bath the slides were immersed in trypsin solution (Biomed-Lublin, Lublin, Poland), then rinsed in PBS solution and stained in Giemsa solution (Thermo Fisher Scientific, *Waltham, MA USA*). After staining they were rinsed again and left to dry. Samples from each culture were digested and stained in two ways (digestion by trypsin and stain by Giemsa):

A. 30 seconds in trypsin and 45 seconds in Giemsa solution (30 sT, 45 sG) and

B. 25 seconds in trypsin and 40 seconds in Giemsa solution (25 sT, 40 sG).

Karyotype analysis

Karyotype, number of metaphases and band resolution of chromosomes were determined using an Olympus BX 61 microscope (Olympus Corp., Tokyo, Japan) connected to an Applied Spectral Imaging software and cytogenetic computer system (Israel Advanced Technology Industries, Herzliya Pituach, Israel).

Statistical analysis

One-way analysis of variance and Student's t-test were performed using SPSS v. 17 software [7].

Results

The spread of chromosomes, the number of metaphase plates on the microscope slide, and the band resolution of the chromosomes were observed (Fig. 1).

The Zendropper was calibrated and the options were set: the height of the stream of water dropped onto the slide, the height of the stream of sample dropped onto the slide, the volume of suspension placed on the slide, and the pressure before the sample is applied. Then smears were prepared in which a similar distribution of cells on the glass slide was obtained irrespective of the type

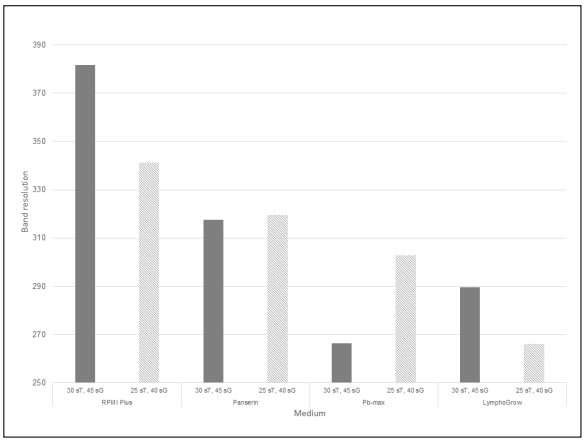

Fig. 1. Band resolution of chromosomes depending on the medium and staining time

of cell cultures (including exact location – x-axis and y-axis) (Fig. 2), as well as a large number of metaphase plates in which the chromosomes spread out to a degree allowing each of them to be evaluated (Fig. 3).

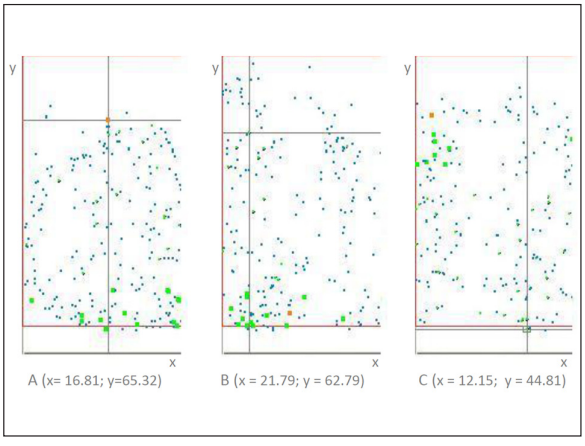

Fig. 2. Distribution of metaphases on microscope slides including location (Olympus BX 61, Applied Spectral Imaging cytogenetic system, magnification 600X)

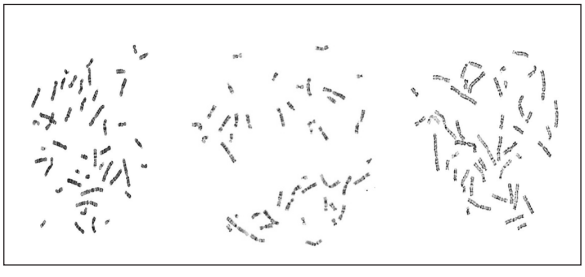

Fig. 3. Spread of chromosomes in metaphases to a degree allowing each of them to be evaluated (Olympus BX 61,

The average number of metaphases obtained after the preparation of 32 smears (N=32) from all 32 cell cultures on all media combined following GTG staining for 30 seconds in trypsin and 45 seconds in Giemsa solution ranged from 21.40 to 124.67. The maximum number of metaphases was 21 and the minimum was 1,057 metaphase plates on the microscope slide. The average number of metaphases obtained after preparation of the smears, taking into account all media used, following GTG staining for 25 seconds in trypsin and 40 seconds in Giemsa solution, was 134.06; the lowest number of metaphases was 6 and the highest was 659 (Table 1).

The mean band resolution obtained after preparation of the slides and analysis of 280 metaphases (N=280) from the 32 cell cultures on all media combined following GTG staining for 25 seconds in trypsin and 40 seconds in Giemsa was 329.91. The lowest band resolution was 150 and the highest was 650. The average band resolution obtained after preparation of the slides and analysis of 291 metaphases (N=291) from the 32 cell cultures on all media combined following GTG staining for 25 seconds in trypsin and 40 seconds in Giemsa was 322.51. The minimum band resolution was 150 and the maximum 575 (Table 2).

Discussion

Karyotype analysis is a very important element in cytogenetics. Preparation of high-quality slides showing long chromosomes that are spread out, metaphase plates that do not overlap, and clearly stained bands can depend on many factors at each stage of the analysis [2,8,9,16]. According to Lim et al. [8], BrdU is used at the cell culture stage to synchronize the cell cycle and to obtain chromosomes with resolution between 450 and 550 BPHS. The concentration and quantity of colcemid added during inhibition of cell division significantly affects the mitotic index and the spread and length of chromosomes. It is also possible to obtain chromosomes with higher resolution at the cell fixation stage, when 0.8% hypotonic sodium citrate is used instead of 0.075 M KCl [8].

Another important factor affecting the quality of the smear is the choice of microscope slide. Some researchers use methods involving placing the suspension on a clean, dry slide or a slide covered with fixative [12]. It is important to use fresh fixative. After some time methanol and acetic acid can have a negative effect on the quality of the smear due to hygroscopic properties. Some laboratories, in order to accelerate or slow down evaporation, have introduced changes in the proportions of methanol and acetic acid (6:1 or 2:1) [11]. The fixative from the suspension dropped onto the slide will evaporate faster in conditions of high temperature and low humidity. According to Rooney, the optimum settings are a temperature of about 20°C and humidity between 40% and 45%. In the case of high humidity of 65% and natural evaporation, the quality of the chromosome spread is considerably lower than in the case of 45% relative humidity.

Table 1. The mean number of metaphases on cell media depending on the duration of *digestion* in trypsin (25 or 30 seconds) and staining in Giemsa solution (40 or 45 seconds)

GTG staining	digestion in trypsin (30 sec) and stain in Giemsa solution (45 sec)					digestion in trypsin (25 sec) and stain in Giemsa solution (40 sec)				
Type of medium	n		SE	Min	Max	n		SE	Min	Max
RPMI plus	8	150.38	40.85	49	340	8	127.63	45.34	39	377
Panserin	8	260.50	124.67	44	1,057	8	168.38	75.75	6	659
Pb-max	8	123.75	43.26	25	352	8	143.88	54.19	34	509
LymphoGrow	8	86.50	21.40	21	196	8	96.38	25.77	14	234
Total	32	155.28	35.20	21	1,057	32	134.06	25.79	6	659

n - number of preparations, -average number of metaphases, SE - standard error, Min - minimum number of metaphases, Max - maximum number of metaphases

Table 2. The mean band resolution of chromosomes on cell media depending on the duration of *digestion* in trypsin (25 or 30 seconds) and staining in Giemsa (40 or 45 seconds)

GTG staining	digestion in trypsin (30 sec) and stain in Giemsa solution (45 sec)					digestion in trypsin (25 sec) and stain in Giemsa solution (40 sec)				
Type of medium	n		SE	Min	Max	n		SE	Min	Max
RPMI plus	80	381.56	10.17	225	600	80	341.25	7.78	200	500
Panserin	68	333.46	11.87	150	650	73	315.75	8.62	150	550
Pb-max	69	301.81	9.63	175	575	71	341.20	11.51	175	575
LymphoGrow	63	291.27	9.00	175	500	67	287.69	10.45	150	500
Total	280	329.91	5.55	150	650	291	322.51	4.93	150	575

n - number of metaphases, - medium band resolution, SE - standard error, Min - minimum band of resolution, Max - maximum band of resolution

For more problematic smears, atmospheric conditions can be artificially modified when the laboratory has equipment making it possible to set and control the temperature and humidity level [5]. According to Hayes, other important factors include the speed at which the suspension is dropped onto the slide and the height from which the cells are dropped, which influence the extent to which the chromosomes are spread, and the incline of the plate [12].

The present study confirmed the effect of selected commercially available cell media on the number of metaphases and band resolution of chromosomes, which had not previously been described, thereby supporting the research hypothesis. In all of the tests performed the cell culture, fixation, slide preparation (automatic method), staining, and number of reagents were identical. The only difference was the medium (RPMI plus, Panserin, Pb-max or LymphoGrow), whose composition had a significant effect on the number of metaphases, and in particular on the band resolution of the chromosomes analysed.

Despite the apparent simplicity of individual procedures such as initiation and inhibition of cell cultures, preparation and staining of slides are not routine. There are many single factors and individual methods affecting the final result of karyotype analysis. The study confirmed the research hypothesis and showed that the commercially available cell media affected the band resolution of chromosomes, which in turn influences the final result of the test. The highest band resolution was obtained from the lymphocyte culture on RPMI plus medium. Using RPMI plus cell medium we were able to create a uniform procedure, making it possible to obtain bands with high resolution, which influences the final result of the test. The use of RPMI plus cell medium for lymphocyte culture also makes it possible to reduce the costs of performing a full karyotype analysis.

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