Distribution and Viability of Peripheral Blood Mononuclear Cells from Imatinib-resistant Chronic Myeloid Leukemia Patients: an *In Vitro* Study

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Abstract

The incidence of Chronic Myeloid Leukemia (CML) in the world is approximately 1.5 cases per 100,000 individuals. The level of resistance to CML treatment, imatinib in Indonesia is relatively high compared to Europe. Culturing CML cells can be used as a model for the determination of pathogenesis of CML, drug efficacy testing, and drug resistance testing. Studies using CML patients' cells to be cultured *in vitro* and the methods used are rarely varied. This study aims to examine the distribution and viability of peripheral blood mononuclear cells from imatinib-resistant CML patients, expected to be a reference for mononuclear cell cultures from CML patients. This study was conducted in June-August 2019 using quantitative descriptive methods. The sample was mononuclear cells isolated from peripheral blood of three imatinib-resistant CML patients at the Hemato-Oncology Polyclinic of Hasan Sadikin Hospital, each of which was cultured *in vitro* using RPMI 1640 for 28 days. Distribution were seen using Giemsa staining, while viability was calculated using trypan blue. Data is processed using Microsoft Excel 2013 and Graphpad. Cell viability decreased during culture. Cell distribution had a different development pattern. Blast cells, eosinophils and basophils had presentation of between 0-5%. The percentage of lymphocyte changed between 11-31%. The percentage of neutrophil changed between 16-41%. The percentage of immature cells decreased, whereas the percentage of monocyte increased. In conclusion, cell viability decreases during the culture. Distribution of cells similar to the initial condition lasted until the 7th day and in the final phase it was only dominated by monocytes.

Keywords: *myeloid leukemia, chronic, cell culture, in vitro, peripheral blood mononuclear cell, cell viability.*

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INTRODUCTION

Chronic Myeloid Leukemia (CML) is a malignancy that attacks white blood cells, characterized by uncontrolled growth of myeloid cells in the bone marrow (Minciacchi, Kumar and Krause, 2021). CML is one of the most common types of leukemia in adult. The annual incidence of CML is approximately 1.5 cases per 100,000 individuals or 15% of all leukemia in adults, the second most after Chronic Lymphocytic Leukemia (Jemal, *et al*., 2010). CML is more common in adults 34-35 years of age in Indonesia (Reksodiputro A, 2015). Management of CML is progressing from conventional chemotherapy such as hydroxyurea and interferon, Stem Cell Transplantation, to target therapy, namely Tyrosine Kinase Inhibitor (TKI), which is now considered to be the most important therapy because of its high efficacy. European LeukemiaNet (ELN) in 2020 recommended TKI as first line therapy for CML (Sumantri, *et al*., 2019; Hochhaus, *et al*., 2020). However, the level of resistance to imatinib and nilotinib as TKI in Indonesia was relatively high (47.69%) compared to Europe (24%) (Jootar, 2012; Jabbour, Cortes and Kantarjian, 2013; Reksodiputro A, 2015).This high level of resistance has contributed to the average survival of CML patients which is only 5-6 years old. Only 20% of patients can survive for up to 10 years or more.

Issues related to CML, both the high incidence, therapeutic resistance, and mortality, require efforts in research approaches from various aspects, including clinical research, community research, and basic research. Focusing on basic research, one of the main assets to be maintained is the ability of CML patients' cells to be cultured *in vitro*.

Culturing cells from CML patients *in vitro* can be used to determine the pathogenesis of CML, drug efficacy tests, and drug resistance tests (Heather, *et al*., 2006). One of the previous *in vitro* studies using cells from CML patients was research by Ravi Bhatia, *et al*. who used CD34+ cells from CML patients. This study used long term culture-initiating cells (LTCICs) in which the number of colony-forming cells (CFCs) was evaluated after 14 days (Bhatia, *et al*., 2003). Other studies using cells from CML patients are using peripheral leukocyte cells, CD-38-, CD133 +, and KCL-22 cultured between 4 days and 14 days (Corbin, *et al*., 2011) (Bono, Dello Sbarba and Lulli, 2018).

Research that uses cells from CML patients to be cultured *in vitro* is still limited and the methods used are still varied, both from the type of cell to the medium used. Optimization is therefore required to obtain a guide method from CML patients in the conduct of cell culture. In Indonesia, no one used CML mononuclear cells in patients with imatinib resistant to be cultured *in vitro*. The objective of this study is to investigate the morphology and viability of cells derived from *in vitro* of peripheral CML mononuclear cells in patients with imatinib resistant over 28 days in the RPMI 1640 medium, this research is hoped to be a guide for mononuclear cell culture in CML patients.

MATERIALS AND METHODS

The study was conducted using a quantitative descriptive design study. The research sample was mononuclear cells from the peripheral blood of three CML patients at the Hemato-Oncology Polyclinic Hospital in Hasan Sadikin Hospital, Bandung, Indonesia. The inclusion criteria for patients were chronic phase CML patients, and resistance to imatinib treatment aged 18-60 years without acute infection. The study was conducted on the basis of an Ethics Permit No.676/UN6.KEP/EC/2019 issued by the Ethics Committee of the Faculty of Medicine, Padjadjaran University and Hasan Sadikin Hospital. The data collection took place between June and August 2019.

The procedure of the study can be seen in Figure 1. The peripheral blood of each patients was drawn up to 10 mL after the patient has signed

the Informed Consent document. Blood was put into the EDTA blood tube and taken to the Culture and Cytogenetic Cell Laboratory of the Teaching Hospital (RSP) Building of the Faculty of Medicine, Padjadjaran University, Bandung to be isolated.

The inclusion criteria in this study was mononuclear cells isolated with Ficoll-Paque Plus solution (GE Healthcare, Chicago, Illinois, USA) and the exclusion criteria was cells that were contaminated with bacteria during the culture process. The patient's peripheral blood, Ficoll Paque Plus and RPMI 1640 (Gibco, Invitrogen, USA), each of which was 10 mL, was centrifuged at a rate of 800 g for 20 minutes at a temperature of 18°C. Peripheral Blood Mononuclear Cell (PBMC) parts were extracted using a pasteur pipette. After that, the cells were washed with PBS (Gibco) and centrifuged at a rate of 400 g for 15 minutes, three times. Then, the cells were washed with RPMI 1640 medium and centrifuged at a rate of 400 g for 10 minutes to obtain clean cells from red blood cells. Mononuclear cells from isolated peripheral blood were counted using trypan blue, and the cells were split into 12 wells for cultures. Cells were cultured in complete RPMI medium (RPMI 1640, FBS 10% (Gibco), Penicillin Streptomycin 1% (Gibco). Cells from each patient were cultured for 28 days, at 37°C, 5% CO_2.

Cells from one well had been seen twice a week for viability and distribution. The sample was homogenized and cell viability was evaluated using trypan blue (Strober, 2015), and a smear was made, followed by Giemsa staining (Brown, 1993). On the 7th day and so on, because some cells have been attached to the bottom of the plate, the cells were given trypsin before the cells were separated, then the cells were counted and centrifuged. Cells from one well were taken and washed with PBS, then centrifuged at 1500 rpm for 3 minutes to get the pellet. The pellet was placed on a glass slide, then smeared using a glass cover and awaited until it dries. Next, the slide was dropped with methanol for fixation and wait for 10 minutes. Then

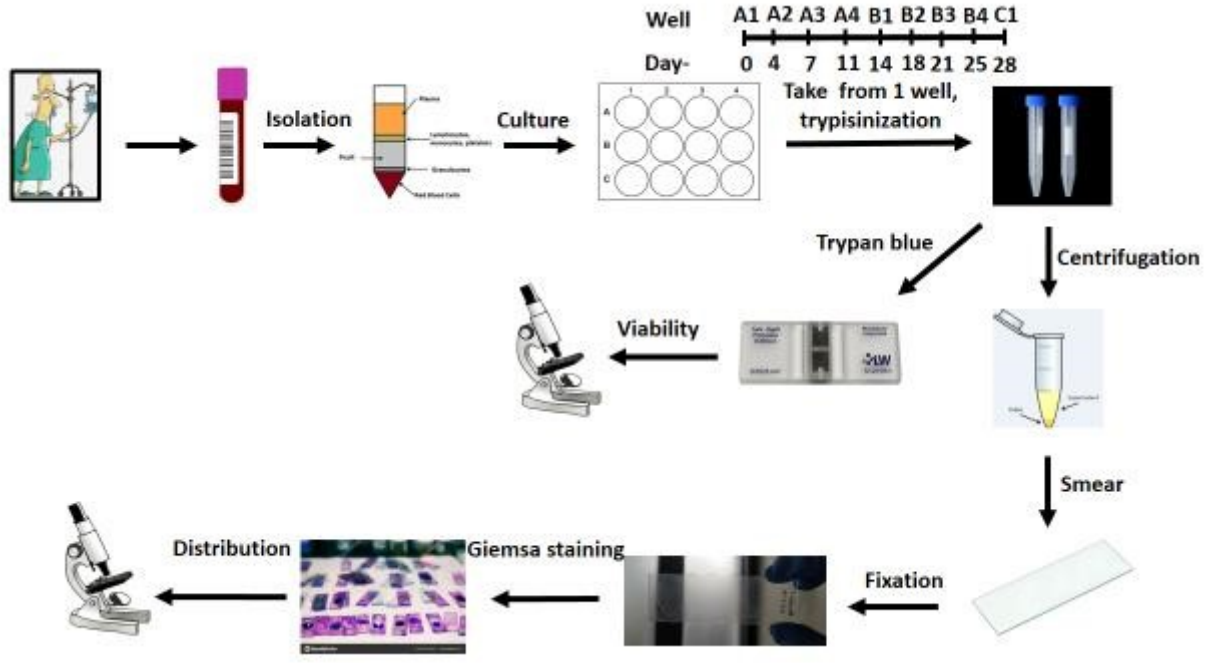

Figure 1. Procedure of the study.

Giemsa staining was done for 30 minutes. After that, Giemsa was washed slowly with running water. The slides were left to dry in the air, then the cells were observed under a microscope. Cells can also be dripped with elastic and covered with cover glass to prevent damage to cells when exposed to immersion oil, so cells were still in good condition if they want to be examined later on.

Differential count was done by observing and identifying morphology of each cell based on the structure and characteristics of mononuclear cells. Cell calculations were carried out in order to obtain 100 mononuclear cells. Cells were categorized into 7 groups: blast cells, immature cells (promyelocytes, prolymphocytes, promonocytes, myelocytes, metamyelocytes), eosinophil, neutrophils, basophils, lymphocytes and monocytes. Data processing was performed using Microsoft Excel 2013 and GraphPad software.

RESULTS

Patient Characteristics

The study was conducted in June-August 2019. Researchers found 6 patients with imatinib-resistant CML at the Hemato-Oncology Polyclinic of Hasan Sadikin Hospital, Bandung. Resistance to imatinib define by molecul response determined from BCR-ABL value in 3-6 months after imatinib therapy. It is resistant if BCR-ABL value neither decreasing/ increasing after the therapy. However, 2 patients refused to participate in this study and 1 patient had an age outside the inclusion criteria. A total of 3 patients were therefore obtained.

Table 1 shows the characteristics of the three patients based on age, sex, baseline hematology, leukocyte count, BCR-ABL status, and treatment status of patients. The three patients were female and were between 18 and 40 years of

Table 1. Characteristics of CML patients in Hasan Sadikin General Hospital.

Characteristics	Patient		
	1	2	3
Age	18	40	33
Gender	Female	Female	Female
Hematological finding			
Erythrocyte (million/μL)	3.63	2.68	5.81
Leukocyte (10^3/μL)	13.89	3.18	186.98
Thrombocyte (thousands/μL)	360	115	1575
Differential Count (%)			
Basophil	0	0	3
Eosinophil	0	5	1
Band neutrophil	0	0	7
Segmented neutrophil	62	42	59
Lymphocyte	33	48	8
Monocyte	2	5	1
Metamyelocyte and myelocyte	0	0	20
Blast	3	0	1
BCR-ABL	(+)	(+)	(+)
Treatment Status			
Type of medicine	Glivec	Glivec	Glivec
Duration of treatment (years)	8	13	2

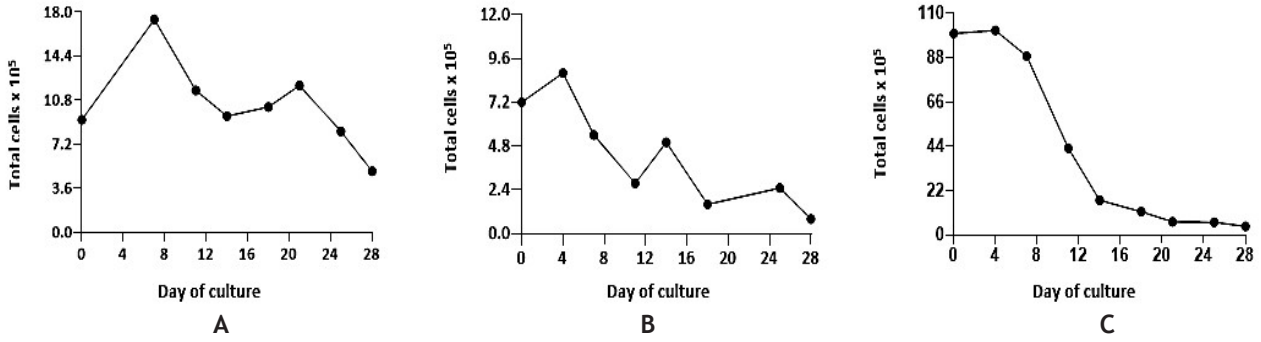

Figure 2. Mononuclear cells viability during the culture. A. Viable cells of patient 1; B. Viable cells of patient 2; C. Viable cells of patient 3.

age. The table shows that the third patient has a very high leukocyte count. From the leukocyte count, neutrophil and lymphocyte predominate in patients 1 and 2, while in patients 3 the dominant cells were neutrophil, metamyelocyte and myelocyte. The BCR-ABL gene was present in all three patients. All three patients received glivec (imatinib) 100 mg per day, which is a first-line CML treatment.

The Viability of Mononuclear Cells Decline during Culture

The blood from each patient was isolated to obtain mononuclear cells and cultured for 28 days. Particularly for patient 3, because the number of cells was very large, researchers only used $10x10^6$ cells. Viability of mononuclear cells in all three patients was calculated using trypan blue. Figure 2 shows that number of living mononuclear cells decreased with time of culture. Although at some point the number of cells increased, in general the number of cells decreased until the 28th day. The number of living cells tended to increase on the 4th day, then tended to decrease on the 7th day until the 28th day.

Figure 3 shows the percentage of mononuclear cells that died during the time of culture. Dead cells were not examined on day 0. Although there was a decrease in the percentage of dead cells of patient 1 (Figure 3.A), butgenerally in patient cell 2 (Figure 3.B), and patient 3 (Figure 3.C), the percentage of mononuclear cells that died increased to the final phase of culture. Even in patient 3 (Figure 3.C), the percentage of cells that on day 28 increased by up to 7 times compared with cells died on day 4.

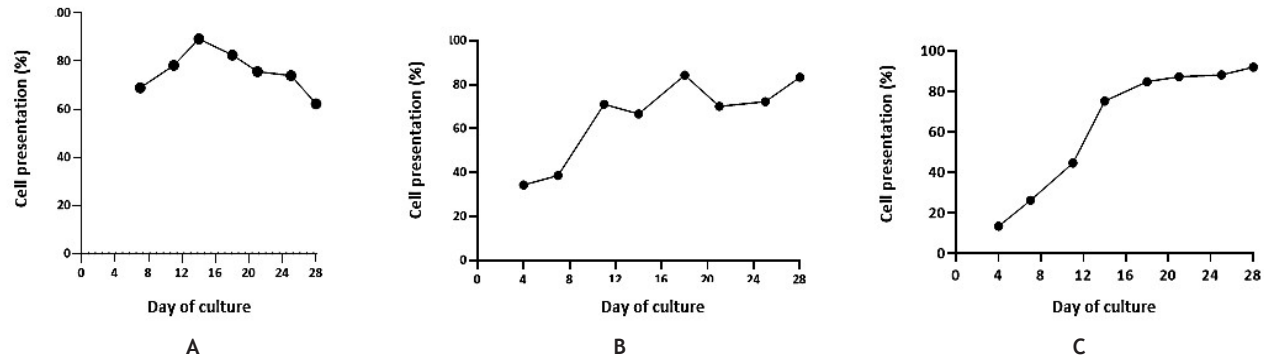

Figure 3. Presentation of dead mononuclear cell during the culture. A. Dead cells of patient 1; B. Dead cells of patient 2; C. Dead cells of patient 3.

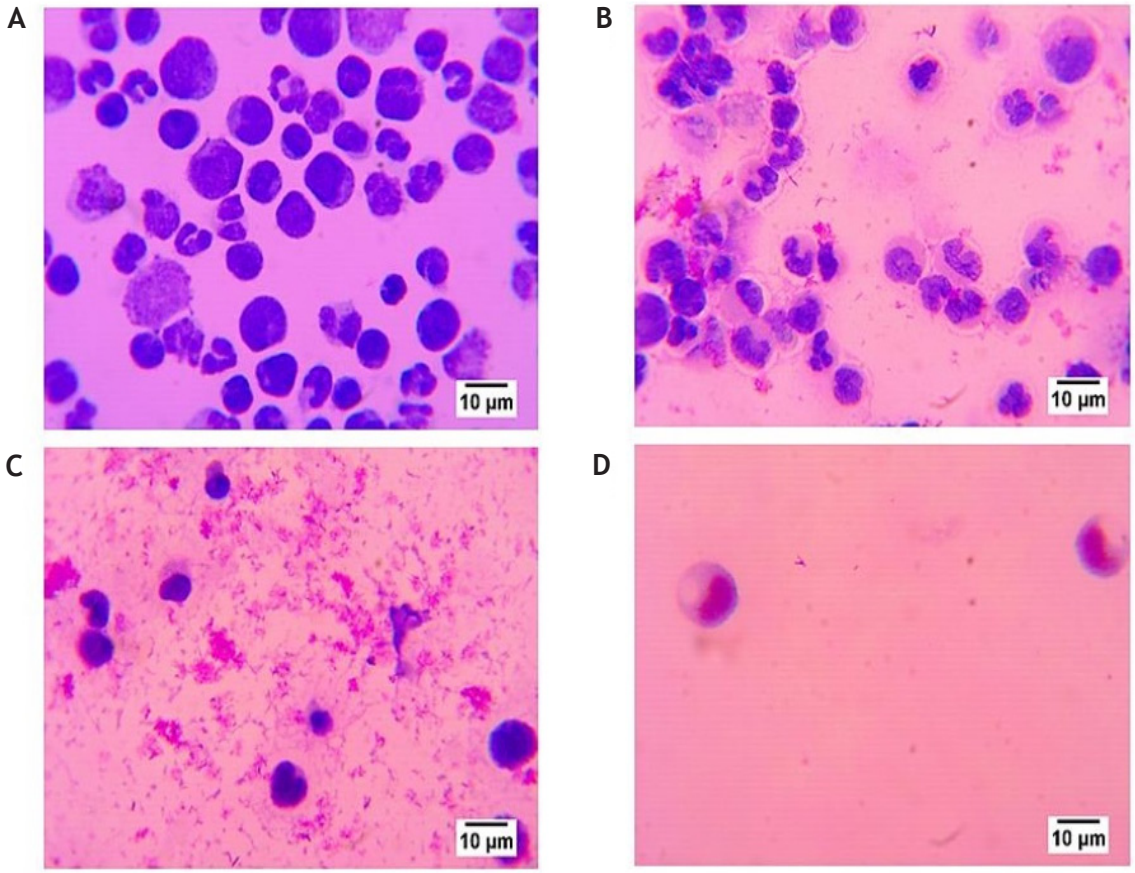

Figure 4. Mononuclear cells of patient 3 microscopically on A. day 0; B. day 7; C. day 14; and D. day 28.

In general, viability of mononuclear cells decreased with time of culture, it can be seen from the graph of decreasing number of living cells and increasing graph of dead cells (Figure 2 (A-C) and Figure 3 (A-C)).

Distribution of Mononuclear Cells Vary during Culture

Figure 4 shows the distribution of mononuclear cells from the peripheral blood of patient 3 seen under a light microscope with a total magnification of 1000x. Various mononuclear cell morphologies could be divided into blast cells, immature cells, eosinophils, neutrophils, basophils, lymphocytes and monocytes. There were still many cells in one field of view on day 0 and it decreased until day 28.

Figure 5 shows a graph of the development of the distribution of mononuclear cells during culture time which is categorized into 7 groups. For blast cells, cells had a fluctuating graph for 28 days of culture, but cell percentages tended to be small and constant around 0-3% (Figure 5.A). For eosinophil cells, it is almost similar to blast cells in which cell percentages tended to be constant with an average of 2% (Figure 5.B). The lowest percentage of eosinophil cells was 1%, while the highest cell percentage was only 5%. For neutrophil cells, the graph went up and down but the percentage tended to decrease until the 28th day, although in some days there was an increase (Figure 5.C). The average percentage of neutrophil cells in all three patients was 28%. The lowest percentage of neutrophil cells was 16%, while the

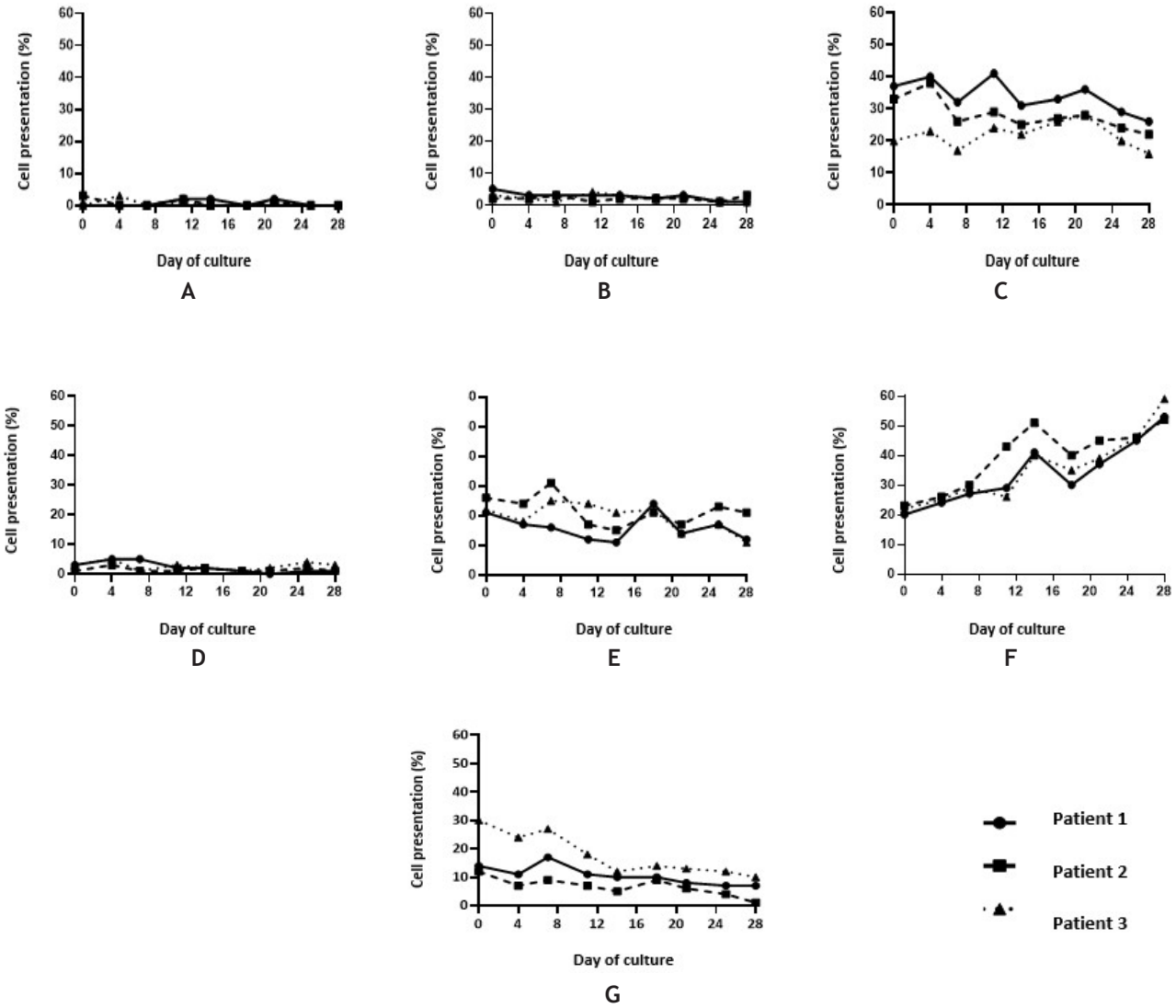

Figure 5. Distribution of mononuclear cells during the culture. A. Blast cells; B. Eosinophil; C. Neutrophil; D. Basophil; E. Lymphocyte; F. Monocyte; G. Immature cells (promyelocyte, prolymphocyte, promonocyte, myelocyte, metamyelocyte).

highest percentage was 41%. Basophil cells were almost similar to eosinophil cells where the cell percentage tended to be small with an average of 2% (Figure 5.D). The lowest percentage of basophil cells was 0%, while the highest percentage of cells was only 5%. For lymphocyte cells, the average cell percentage was 19%. The lowest percentage of lymphocyte cells was 11%, while the highest percentage was 31% (Figure 5.E). For monocyte cells, the graphic pattern tended to increase with culture time (Figure 5.F). At the beginning of culture, the percentage of monocyte cells was only about 20%, but on the last day of culture, the percentage of monocyte cells could reach 59%. In inverse proportion to monocytes, immature cells such as promyelocytes, myelocytes, and metamyelocytes had a high percentage tendency in the early days of culture, but over time the culture,

the percentage of immature cells decreased (Figure 5.G). Immature cells tended to go down on day 4, then went up on day 7, but decreased until day 28. Percentage of immature cells on day 0 could reach 30%, but on day 28 there was 1% cell left.

DISCUSSION

The data was collected from 3 CML patients resistance to imatinib that met the inclusion criteria. The third patients showed a very high leukocyte count with dominant cells, which were neutrophil, metamyelocyte and myelocyte. In contrast with patient 1 and 2 with leukocyte count predominantly neutrophil and lymphocyte. The third patient has not been in control for the treatment more than 3 months so that it is likely contributed to the patient's blood results. All patients show BCR-ABL (+) with different duration of treatments.

There were variations in the decline of viability of mononuclear cells during culture. In general, the number of cells decreased until the 28th days. This corelates with the results in Figure 3, that showed the percentage of dead mononuclear cells during the culture. This results is in accordance with previous studies that carried out in leukocyte cell culture in CML patients and found that 5 out of 8 patients had cell viability that decreased with the time of the culture (Golde, Byers and Cline, 1973). From the results in Figure 2, it can be seen that generally living cells survived as the initial condition until the 7th day, and finally the number of living cells decreased.

The morphology and distribution of mononuclear cells could be identified and categorized into 7 groups: blast cells, immature cells (promyelocytes, prolymphocytes, promonocytes, myelocytes, metamyelocytes), eosinophil cells, neutrophils, basophils, lymphocytes and monocytes. When viewed from the development of cell types during the observation period, different dynamics between cell types were observed. Blast, eosinophils and basophils had a small and constant percentage (Figures 5.A, 5.B, and 5.D). Lymphocyte undergo changes, wherein the percentage of cells ranges from 11-31% (Figure 5.E). The percentage of neutrophil cells changed between 16-41% (Figure 5.C). The percentage of immature cells decreased, whereas the percentage of monocyte cells increased with culture time (Figures 5.F, and 5.G).

In the research results, it was found that the blast cells only had a percentage between 0-3 % (Figure 5.A). This result is related to the inclusion criteria for patients who are chronic phase CML patients, where the grouping of these phases is categorized based on the percentage of blast cells in patients. CML patients are said to be in the chronic phase if they have blast cells of less than 10% (Thompson, Kantarjian and Cortes, 2015; Bruneau and Molina, 2020), which is a criteria that is still used to this day (Radich, *et al*., 2018; Osman and Deininger, 2021; Jabbour and Kantarjian, 2022).

Eosinophil had a relatively small percentage, between 1-5% (Figure 5.B). Based on the literature, eosinophils can only be cultured in the presence of cytokines that can inhibit the process of apoptosis such as IL-3, IL-5, and GM-CSF (Wong and Jelinek, 2013). Without these cytokines, eosinophil can only survive a few days during culture. The addition of IL-5 can increase eosinophil survival, but in this study IL-5 was not given. Furthermore, eosinophil are also unable to divide in such a way that they cannot proliferate after being isolated from human peripheral blood (Cotter, 2001). These things that cause the results of eosinophil graph have a percentage of cells that tend to be small and constant. Basophils also cannot divide because they are the final stage of the differentiation process. This also supports basophile cell charts with a small and constant percentage (Figure 5.D).

Immature cells (promyelocytes, prolymphocytes, promonocytes, myelocytes, metamyelocytes) had a declining percentage

(Figure 5.G). Promyelocytes and myelocytes have the ability to divide, evidenced by the incorporation of thymidine marked with tritium into the DNA of the nucleus and have been verified in direct observations in the culture process (MacQueen, *et al*., 2019). The stages of myeloid cell maturation have different kinetic properties that have been studied *in vitro* using radioisotope label studies. It was found that it takes about 5 days from myelocyte cells to mature neutrophil cells. When linked to this study, immature cells can divide into neutrophils. This makes the percentage of immature cells decreased with time of culture (Figure 5.G) and the percentage of neutrophil cells increased (Figure 5.C).

Nevertheless, when neutrophil is cultured *in vitro*, it can spontaneously undergo apoptosis (Kirschnek, *et al*., 2011). Neutrophil death due to apoptosis can be triggered and increased by a number of extracellular or intracellular stimuli such as cytokine inflammatory mediators, reactive oxygen species (ROS) and nitric oxide (Watson, 2002). Neutrophils are cultured culturally *in vitro* in Iscove's MDM medium for 20 h experienced secondary necrosis (Hannah, *et al*., 1998). Due to the short life span of neutrophils and their fragile nature, so that even though the percentage of neutrophil cells had increased, if they had been cultured for a long time, the percentage of neutrophils would have decreased.

Lymphocyte experienced a percentage change during the time of culture, where the percentage of cells was between 11-31% (Figure 5.E). Several previous studies have suggested that culturing lymphocyte in RPMI medium no more than 24 h, because after that cells can undergo apoptosis (Bulla, *et al*., 2014). This is consistent with the lymphocyte cell chart which tended to decrease with culture time. If you want to culture lymphocytes for longer periods of time, you need additional conditions related to the medium and supporting cells.

Monocytes increased in percentage over cultivation (Figure 5.F). This is consistent with previous studies that conducted primary culture of monocyte *in vitro* within 4 months. In this study, monocyte cells had a tendency to increase with culture. In addition, monocyte cells also increased in size and could differentiate into macrophages, fusiform and epithelioid forms (Zuckerman, Ackerman and Douglas, 1979). Overall, cell distribution can survive as the initial conditions until the 7th day, after that day, when most cells had decreased percentages except for monocyte which actually increased percentage.

Limitations in this study include the number of patients that is only 3. This is due to the limited time of the study while each patient cell must be cultured for 28 days. In addition, calculations using trypan blue also provide limitations, where the calculation and division of cells into 12 wells is not exactly the same. Another limitation in this study is that the instruments available are only light microscopes with a maximum magnification of 1000x. In fact, it is easier to identify mononuclear cells using an electron microscope, especially to distinguish immature cells, but the only facility available is a light microscope. In addition, macrophages in this study were considered the same as monocytes because trypsin is administered to separate cells. The medium was changed only once during the study due to the limited capacity of the well, so there is a possibility to fight for nutrition so that many cells died. This research is not duplicate or triplicate so that it also one of the limitations.

CONCLUSION

From this study, it can be concluded that the viability and distribution of peripheral blood mononuclear cells in imatinib-resistant CML patients cultured *in vitro* using RPMI 1640 medium changes over 28 days of culture. Cell viability decreases with time of culture. The

distribution of cells similar to the initial condition lasted until the 7th day, then in the final phase it was only dominated by monocyte cells.

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