CYTOSPECTRAL CHARACTERIZATION OF STAINED LEUCOCYTE TYPES

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Little is known about the spectral characteristics of leucocytes stained by routine methods and their values in blood cytodiagnosis. In this work a new method was developed for measuring color differences per cell between different cells, and the applicability of this method in characterization of leucocyte types was examined. In this new method the relative color spectrum was measured over the range of 370 nm to 700 nm as the ratio of the absorption at each wavelength to the maximum absorption ($A/A_{\max}$, %). The relative color spectrum was obtained from the absorption curve by a wavelength-scanning method using a double-beam microspectrophotometer with a projection beam area larger than the cell size. The error due to staining was least when blood cells are stained by a modification of the method of Wright. Several types of human and animal leucocytes were distinguished on the basis of their relative color spectra, and these spectral differences seemed suitable for blood cytodiagnostic purposes, because a fixed spectral pattern was obtained for most leucocyte types, even including lobated-nuclear neutrophils. However, it has not yet been possible to distinguish eosinophils from pseudo eosinophils in fowl blood or monocytes from some lymphocytes.

Many staining methods have been developed for cytodiagnosis of various blood cell types. Methods for identifying blood cell types using polychromatic stain, such as the method of Giemsa (Romanovsky 1891, Giemsa 1904) or its modifications (May, Grünwald 1902, Wright 1902, Pappenheim 1911) are empirically effective. Using these methods cytodiagnosis is possible due to differences in size, shape and staining intensity of different cell types. Attempts to differentiate eosinophils by differences in color have been unsuccessful (Matsueda 1954). Thus, except for erythrocyte species, which can be distinguished by differences in the absorptions of heme and hemoglobin derivatives (Thorell 1947, 1955, Jope 1949, Metcalf 1949, Wilkins 1953, Nakao 1957, Seno 1958) blood cytodiagnosis on the basis of differences in color, especially of stained cells, is still not possible.

Accordingly we attempted to measure the color spectrum per leucocyte using an efficient cytospectrometric method and to compare spectral differences in staining of various leucocyte types for use in blood cytodiagnosis. First we tried to demonstrate color differences per individual cell in different types of leucocytes in a leucocyte population. For this we developed an effective method for measuring

Dedicated to Professor Hideo Takamatsu in memory of his retirement.

the relative color spectrum per cell. We distinguished several types of leucocytes by their characteristic relative color spectra. This spectral classification of blood cell types should be useful for cytodiagnostic purposes.

MATERIALS AND METHODS

Blood preparation: Samples of 2 ml of peripheral venous blood were obtained by syringe and immediately mixed with 1 drop of Anglot/ET (Nippon Shoji Co. Osaka) containing 8% EDTA complex and 0.001% heparin to prevent clotting. Blood samples were taken from man and animals such as cow, pig, guinea pig and fowl. To separate the leucocyte from erythrocytes, the blood was centrifuged at 1,000 r.p.m. for 5 minutes, and then the upper layer of the pellet was diluted with four or five volumes of the clear supernatant blood plasma. The resulting cell suspension was smeared as a thin layer on a slide-glass. The smear was immediately dried in air, fixed in methanol and then dried again. This specimen was stained by the Giemsa (Giemsa 1904), May-Giemsa (Pappenheim 1911) or Wright (Wright 1902) method for blood cytodiagnosis.

Optimal staining was achieved by careful examination of the dye concentration and the time for staining at room temperature, because the maximum absorptions of polychromatic dye components vary (Lillie-Roe 1942). In this study Giemsa staining was performed using a ratio of 2 ml of 10 mM phosphate buffer (pH 6.4) to 3 drops of Giemsa solution (Merck) for 40 minutes, May-Giemsa staining was achieved by treatment with 1 ml of May-Grünwald solution (Merck) for 3 minutes, addition of 1 ml of the buffer for 3 minutes and then restaining by the Giemsa method as described above. Wright staining was achieved by treatment with 1 ml of Wright solution (Merck) for 2 minutes, and then addition of 1 ml of buffer for 5 minutes. Other procedures were carried out by routine methods as described elsewhere (Lillie 1948).

Cytospectrophotometry: Before measurements, the absorptions of the slide-and cover-glasses in the range of wavelengths (λ s) of 350 nm to 700 nm were examined to prevent optical errors due to the photometric system. A micro-spectrophotometer of the Zeiss UMSP-1 type or sometimes of the Nikon-Vickers M 86 type was used for both λ - and area-scannings (Caspersson 1955, 1962). It was not possible to measure the curve for the absolute optical density (OD), because the size and shape of cells was so variable and irregular that it could not be matched by a round micro-beam of fixed diameter (Patau 1952). Actually the photometer used was equipped with a projection beams of various diameters at the objective plane and the measuring diaphragm, as shown in Table 1. The relative absorption is easily obtained by λ -scanning, but it differs in individual cells, depending on the diameter of the micro-beam which covers the whole cell on the measuring diaphragm in the objective plane. Therefore, a curve of the relative absorption (RA) was obtained as the percentage ratio ($A/A_{\max}$, %) of each absorption (A) to the maximum absorption ($A_{\max}$), even when dimensions of the beam were larger than those of the cell. The relative absorption curves, showing the relative color per cell, obtained using beams of 12.5 μ to 25 μ diameter were compared. The curves obtained with similar and different cell types were also compared. When the RA (%) was replaced by the OD previously integrated

FIG. 1-a

FIG. 1-b

FIG. 1-c

TABLE 1. Conditions for measurements

	λ -scanning	Area-scanning
Objective $\times$ Projection eye piece	32×10	32×100
Measuring diaphragm in object plane (μ)	1) 12.5 2) 16 3) 20 4) 25	2
Engraving on measuring diaphragm (mm)	1) 4 2) 5 3) 6.3 4) 8	6.3
Line jump (μ)	—	2

by area-scanning at the λ of A max, the absorption curve, which was proportional to the RA curve, could be reconstructed by OD measurement at only the λ of the A max. The absorption curves obtained for different cell types were also compared.

RESULTS

I. Measurement of the relative absorption (RA) curve

The absorption curve were obtained by λ -scanning with a beam of sufficient diameter to cover the whole cell. Thus, in the T% curves of leucocytes shown

FIG. 2. The relative absorption curves of human leucocytes calculated from Fig. 1-b. Abbreviations are as in Fig. 1-a.

- FIG. 1-a. T% curves of leucocytes of fowl blood stained by the May-Giemsa method. Lym: lymphocyte, Mon: monocyte, BL: basophil, NL: neutrophil, EL: eosinophil, bg: background transmittance
- FIG. 1-b. T% curves of leucocytes of human blood stained by the Wright method.
- FIG. 1-c. T% curves of leucocytes of human blood stained by the Giemsa method.

FIG. 3. Visible light absorption spectra of dye solutions used for blood cytodiagnosis. Solid line: Wright solution (Merck, Art. 1383, No. 2527022) at $160\times$ dilution, broken line: Giemsa solution (Merck, Art. 9204, No. 2549770) at $500\times$ dilution, dotted line: May-Grünwald solution (Merck, Art. 1424, No. 0201718) at $180\times$ dilution.

in Fig. 1, the background causes interference with the sample absorption (most in Fig. 1-a). In practice, the background error was least in cell stained by the Wright method (Fig. 1-b) and most in those stained by the May-Giemsa method (Fig. 1-a). The difference in the colors of various leucocytes was also greater using the Wright method than using the Giemsa method (Fig. 1-c). Therefore, in subsequent studies specimens were stained by the Wright method.

The chief problem in this work was how to compare relative absorption (RA) curves obtained using beams of various sizes which usually did not fit the size and shape of the cells well. A RA curve was required which was independent of the beam size. For this purpose we adopted the RA, that is the percentage ratio of the absorption at each wavelength to the maximum absorption ($A/A_{\max}$, %) instead of the direct transmittance shown in Fig. 1. In the RA spectra of stained blood cells the $A_{\max}$ is 100%, and the A at 370 nm (A_{370}) is about 0%, as shown in Fig. 2. This low value of A_{370} closely resembles the low absorption of dye solutions at below 400 nm shown in Fig. 3. In mammalian erythrocytes, where A_{370} is influenced by the absorption near 415 nm due to oxyhemoglobin (Van Assendelft 1970), the 0% at A_{370} may be replaced by the A at 700 nm.

In practice, as the size of individual cells varies, the corresponding beam size is also varied according to the principle of cytophotometry (Caspersson 1955, 1957), when the whole cell is scanned by λ . So, the RA curve should be ascertained by measurements using various sizes of projecting beam. When the RA curve for a single cell is measured using various beam sizes which are larger

FIG. 4-a. RA curves of a pig lymphocyte measured with two different beam diameters of $12.5\ \mu$ and $16\ \mu$.

FIG. 4-b. RA curves of a bovine monocyte measured with two different beam diameters of $16\ \mu$ and $20\ \mu$.

FIG. 4-c. RA curves of a bovine neutrophil measured with two different beam diameters of $16\ \mu$ and $20\ \mu$.

FIG. 4-d. RA curves of a bovine eosinophil measured with two different beam diameters of $20\ \mu$ and $25\ \mu$.

than the whole cell and cover it fully, the curves are found to coincide well for single cells such as lymphocytes, monocytes, neutrophils or eosinophils, as shown in Fig. 4.

II. Reliability and specificity of the RA curve

To test the reliability of the RA curves, the RA curves for leucocytes from different sources identified by the usual cytodiagnostic procedure were measured. As shown in Fig. 5-a, 10 lymphocytes from 4 pigs gave similar RA curves and, as shown in Fig. 5-b, 5 neutrophils with various extents of nuclear lobation from 5 human blood specimens also gave similar RA curves. Similar curves were also obtained with other kinds of leucocyte populations, such as lymphocytes, monocytes, eosinophils and basophils of human blood. These results show that different types of leucocytes show characteristic RA curves, even when the leucocytes are obtained from different cell populations.

The RA curves of leucocyte populations from different kinds of animals were also measured by the scanning method. The RA curves of leucocyte populations

FIG. 5-a

FIG. 5-b

FIG. 5-a. RA curves of 10 pig lymphocytes measured with a constant beam size of 12.5 μ .

FIG. 5-b. RA curves of 5 human neutrophils measured with a constant beam size of 16 μ .

TABLE 2. *Spectral types of leucocytes*

Type	A max (nm)	RA600 (%)
I	550-536	>50
II-a	535-525	>50
II-b	535-525	<49
III-a	524-516	>50
III-b	524-516	<49
IV	515-510	

from both human and fowl blood showed maximum absorption at just over 500 nm and ranged from 370 nm (A₃₇₀ is 0%) to over 700 nm. The curves can be roughly divided into several classes on the basis of the wavelength of their maximum RA (100%). Moreover, the profiles of the RA curves of the leucocyte populations of man and fowl appear different at above 570 nm. These two parameters were usually different in leucocyte populations of different vertebrates and details of these differences will be published in the following report (Takeuchi, Yamada 1976).

On examination of the leucocyte populations of different vertebrates the wavelength of the A max was found to range from 510 nm to 550 nm and the A max of leucocyte populations of vertebrates lower than birds was at shorter wavelengths. Therefore, the spectra were divided into several types on the basis of the position of their A max, as follows: type I, A max at 550-536 nm; type II, A max at 535-525 nm; type III, A max at 524-516 nm; and type IV, A max at 515-510 nm, as shown in Table 2. The RA at 600 nm (RA600) was adopted as a parameter to indicate eosinophils, because eosinophils mainly have RA600 values of below 49% whereas no human monocytes have RA600 values in this range, as shown in Table 3. On the basis of these two criteria the basophils of both fowl and human blood are classified as type I and separated from the other leucocyte types in the blood. But the basophils of guinea pig blood are classified as type II-a. The monocytes of human blood are widely distributed in both type II and type III, but the distributions of subtypes of monocytes are limited. Unlike human monocytes, fowl monocytes are of type III-b and fowl eosinophils of type IV. The so-called pseudo eosinophils (Cullen 1903, Forkner 1929) in fowl blood are of type III-b, like human eosinophils. Thus, it seems possible to classify leucocytes into several types on their cytospectral characteristics. However, their spectral patterns do not agree very well with the present cytodagnostic classification of leucocytes.

TABLE 3. *Correlation between spectral types and cell types*

Animal	Spectral types					
	I	II-a	II-b	III-a	III-b	IV
Man	B	LM		MN	NE	
Guinea pig	L	BLM		N	E	
Cow	BL	LM		NE	E	
Fowl	B	L	L	T	MP	E

B: basophil, N: neutrophil, E: eosinophil L: lymphocyte,
M: monocyte, P: pseudo eosinophil T: thrombocyte

III. Absorption spectra obtained by a combined scanning method

It is not easy to obtain the absolute absorption spectrum per cell. But the RA curve can be obtained simply by λ -scanning and the absorption at the λ of the relative A max (100%) can be measured by routine area scanning (Caspersson 1955, 1962). When the A max of 100% in the RA curve is replaced by fA (total A max) at λ max integrated by area-scanning, and the other RA values (A/A max, %) are successively replaced by $fA \times A/A$ max, the RA curve can be transformed into an absorption curve showing the relative optical density at each wavelength. Using this combination, the RA curve can be adequately replaced by $A \times A/A$ max. The absorption curves for human and fowl leucocyte populations corrected in this way are shown in Fig. 6-a and Fig. 6-b, respectively.

There seem to be three types of A max: a higher type ($A > 6 \times 10^{-2}$), an intermediate type ($A < 6 \times 10^{-2}$) and a lower type ($A < 4 \times 10^{-2}$). In the human leucocyte population, eosinophils and basophils show the higher type, neutrophils and monocytes show the intermediate type, and lymphocytes show the lower type. In the fowl leucocyte population, in which the thrombocytes and pseudo eosinophils are different from human leucocytes, the basophils and pseudo eosinophils as well as the eosinophils show the higher type, the monocytes show the intermediate type, and the lymphocytes and thrombocytes both show the lower type. Similar estimations were made on neutrophils of human blood, which vary in nuclear lobation. The RA curves of human neutrophils showed a similar pattern with an absorption peak at

FIG. 6-a. Absorption curves (fA curves) of human leucocytes in which RA curves such as those in Fig. 2 are replaced by fA as measured by area-scanning. Abbreviations are as in Fig. 1-a.

FIG. 6-b. Absorption curves (fA curves) of fowl leucocytes obtained as described in Fig. 6-a. PEL: pseudo eosinophil, Thr: thrombocyte. Other abbreviations as in Fig. 1-a.

FIG. 7. Absorption curves ($\int A$ curves) of human neutrophils with variously lobated nuclei: I, one lobe; II, two lobes and IV, four lobes.

about 520 nm and a slight shoulder at about 600 nm. The curves for individual neutrophils were very similar and their A values at each wavelength were nearly constant, as shown in Fig. 7, even though the individual cells showed considerable variation in the extent of lobation of their nuclei.

From this constancy of the absorption pattern observed in a single cell species, it seems possible to use the characteristic absorption pattern as an indicator of leucocyte species, instead of a subjective method in cytodiagnosis. The absorptions of different species of leucocytes in populations from both man and fowl are in general correlated with the leucocyte species recognized in cytodiagnosis.

DISCUSSION

Most of the dye solutions commercially available for use in routine methods for staining blood cells have complex spectra, as shown in Fig. 3. However, preparations of Giemsa or its modified solution have rather simple spectra with two absorption maxima and only show minor differences in absorption at higher wavelengths. In the present work it was found that even when the absorption of the dye solution was constant an error was introduced by the background absorption of the slide-glass which seemed to differ in each method and was only negligible when specimens were stained with Wright solution (Fig. 1-b). This background absorption was minimal when blood cell smears were subjected to the Wright method at pH 6.4. Moreover, the differences in the absorptions of different types of leucocytes were greater using the Wright method than the Giemsa method. Thus the Wright method (Wright 1902) was adopted as being most suitable for the present spectrophotometric studies. The eosinate dye complex in commercial products is reported to be variable (Lillie-Roe 1942). So, Wright solution with a fixed spectral pattern (Fig. 3) was used throughout, taking care to avoid dye variability.

Microphotometry of nucleic acids using the Feulgen method has been used to estimate the relative contents of nucleic acids during cell growth (Caspersson 1955, 1957, Naora 1955, Seno 1958, Fujita 1971) and DNA has mainly been estimated on lymphoid cells (Hale-Wilson 1961, Killander-Rigler 1965, Jobst-Sandritter 1965, Miyoshi-Kono 1965, Garcia-Iorio 1966, Queisser-Sandritter 1966, Mayall-Mendelsohn 1970). In some studies the two-wavelength method (Mendelsohn 1961) has been used. There are a few reports on assay of DNA (Müller 1964) and phosphatase (Van Der Ploeg-Van Duijn 1968) in granulocytes. In studies on leucocytes, azure dye has also been used to counterstain RNA and differentiate it from DNA by its metachromatic reaction (Michaelis 1947, Kurnick 1950, Walker 1959, Garcia 1966, Shea 1970). However, using a single cationic dye, the color differences between different types of leucocytes were not sufficient for application of the method to blood cytodiagnosis. Giemsa and its modified solutions contain mixtures of methylene blue, azure and its eosinate (Lillie-Roe 1942), so that various structures as well as nucleic acids can be stained. Thus, staining with a mixed-dye such as Giemsa has been empirically the most effective in determining blood cell types. But the effectiveness of mixed-dyes for cytodiagnosis is not due to the additive effects of basic dyes in staining nucleic acids, because mixed-dyes react competitively on each cell structures, as described elsewhere (Michaelis 1947, Singer 1952, Walker 1959).

To compare the absorption spectra of different blood cells it was necessary to have at least a fixed expression of an absorption unit, because the absolute extinctions of individual cells varied. This requirement was satisfied by calculating the percent relative absorption ($A/A_{\max}$, %) of each absorption (A) to the maximum absorption ($A_{\max}$), as described in the results, section I. A similar expression has been used for Feulgen-stained leucocytes (Garcia 1966), but has never been applied in blood cytodiagnosis. It is very useful to be able to express 0% in the wavelength range as a standard of the relative absorption spectrum. Fortunately using the Wright method, A at 370 nm (A_{370}) is close to 0, and A_{370} is negligible in carefully prepared specimens. Accordingly, the A_{370} can be placed at the 0% level. In the case of erythrocytes, it seems difficult to fix A_{370} at 0% because A_{370} is influenced by the absorption near 415 nm due to oxyhemoglobin (Van Assendelft 1970). With mature erythroid cells, A at 700 nm (A_{700}) instead of A_{370} may be fixed at 0%, but with immature or nucleate erythroid cells we found that A_{700} is rather higher than A_{370} (Yamada *et al.* 1976). In comparing the relative color spectra ($A/A_{\max}$ from 370 nm to 700 nm) of leucocytes a good coincidence was obtained with single cells using different sizes of projecting light beam. The spectra of different individual cells of the same leucocyte species also coincided well. These data show that the method of measuring $A/A_{\max}$ curves is applicable to leucocytes, even when the cells are of various sizes and shapes.

Using this method of cytospectrophotometry, different types of leucocytes in mammalian and fowl blood were examined. A close correlation was found between the cytodiagnostic and spectral classifications. The correlation between similar types in different species was not so good, but in a given animal species, different individual cells of a single type showed the same characteristic spectrum. Thus, leucocytes can be characterized on the basis of their spectral types.

Moreover, the relative color spectrum can be multiplied by the replacement

with a A integrated a certain wavelength, as shown in Fig. 6-a and b. Using this modification the spectra can be further classified into three types; a higher, an intermediate and a lower type. With this additional classification on the A-dimension, it seems possible to distinguish three subtypes of RA curves. In this way the classification can be expanded to differentiate more detailed and similar RA curves of different cell types, such as human and fowl leucocytes (Fig. 6).

In some cases it was not possible to differentiate leucocytes of a single animal species, or more especially of different animal species, but even in the routine method for blood staining it is not possible to identify some types of leucocytes, such as those described as heterophilic leucocytes (Maximow 1937, Bloom-Fawcett 1975). Pseudoeosinophils have been distinguished from eosinophils in fowl blood (Cullen 1903, Forkner 1929), but it was not possible to differentiate them by the present method. Among guinea pig leucocytes heterophilic leucocytes may be confused with neutrophils and among human leucocytes the spectral type of eosinophils resembles that of some lymphoid cells. Due to these disadvantages the present spectral classification can still not be used alone for cytodiagnosis of all leucocytes, but must be used in combination with the classic method of staining with polychromatic dyes.

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