A COMPARATIVE CYTOSPECTRAL STUDY ON VERTEBRATE LEUCOCYTES STAINED BY THE WRIGHT METHOD

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A new method was developed for cytospectral characterization of leucocytes after staining with Wright solution. The spectral types of various vertebrate leucocytes were compared by this method. To characterize vertebrate leucocytes their absorption maximum (A max) and relative absorption (RA) spectra (A/A max, %) were recorded. From the values of A max and the absorptions at 600 nm (RA 600) and 460 nm (RA 460), the RA spectra were divided into 7 types (I, II-a, II-b, III-A, III-a, III-b, IV). The leucocyte types of individual species could be classified into certain types, but these types differed in different species. However, even in different species, most thrombocytes were classified as type III-A, mammalian eosinophils as type III-b and those of lower vertebrates as type IV. In general the spectral types changed progressively from higher to lower animal species. The blood of lower vertebrates seemed to contain immature cells judging from the RA spectra of these cells. From our data this spectral classification seems promising for comparison of vertebrate leucocytes and should be useful in their cytoanalysis.

Most cytophotometric studies on leucocytes have been made on the DNA of lymphoid cells (15, 17, 20, 24, 31, 32, 35) and in some studies the two-wavelength method (34) has been used. There have also been a few studies on the assay of DNA of granulocytes (37) and phosphatase of neutrophils (42). Cytofluorometry has also been used for quantitation of DNA in leucocytes (24).

However, little is known about the color spectra of different types of leucocytes stained by standard method, such as Giemsa staining (16) or Wright staining (45). Recently we reported studies on the characterization of leucocyte types by cytospectral analysis (46). Since the time when polychromatic stains, such as Giemsa and Wright stains became available for blood cytoanalysis, there have been many morphological studies on vertebrate leucocytes, but with these stains it is difficult to find a suitable standards for comparing the leucocyte types of different animals, and especially lower vertebrates. Therefore, in this work we attempted to compare leucocytes of higher and lower vertebrates on a cytospectral basis. For comparative purposes we also tried to classify vertebrate leucocytes into various spectral types.

MATERIALS AND METHODS

Blood preparation: Blood samples of about 2 ml were drawn into a syringe

with 1 drop of Anglot/ET (Nippon Shoji Co., Osaka) containing 8% EDTA and 0.001% heparin to prevent clotting. These samples were taken from the veins of human and big animals, such as cow (*Bos taurus*, Gmelin), pig (*Sus domesticus*, Brisson) guinea pig (*Cavia cobaya*, Schreber) and fowl (*Gallus domesticus*, Brisson) and from the heart of small animals, such as the gecko (*Gekko japonicus*, Duméril et Bibon), lizard (*Eumeces latiscutatus latiscutatus*, Hallowell), snake (*Elaphe climacophora*, Boie), frog (*Rana nigromaculata nigromaculata*, Hallowell), tortoise (*Clemmys japonica*, Temminck et Schlegel), tuna fish (*Thunnus orientalis*, Temminck et Schlegel) and eel (*Anguilla japonica*, Temminck et Schlegel). To separate leucocytes from erythrocytes, the blood was centrifuged at 1,000 r.p.m. for five minutes, and the upper layer of the pellet was diluted with four 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 Wright's method (45) for blood cytoanalysis. Optimal staining was achieved when the specimen was treated with 1 ml of Wright solution (Merck, Art. 1383, No. 2527022) for 2 minutes, and then 1 ml of 10 mM phosphate buffer at pH 6.4 for 5 minutes. Other procedures were carried out by routine methods as described elsewhere (27). A great variety of terms has been used in cytoanalysis of the blood of vertebrates for different animals and by different authors. The terms used in this paper are consistent with those used in the textbook of Downey (23).

Cytospectrophotometry: In the previous report on absorption analysis of leucocytes types (46), the wavelength (λ) scanning method was employed for measurement of leucocytes free from other cells on the objective plane of a microspectrophotometer. For this purpose a Zeiss UMSP-1 type instrument was used for λ scanning on the projection beam with various diameters at the objective plane, as described elsewhere (6). The relative absorption (RA) was obtained as the percentage ratio ($A/A_{\max}$, %) of each absorption (A) to the maximum absorption ($A_{\max}$). The $A_{\max}$ was empirically chosen in the range from 550 nm to 510 nm in the leucocyte populations, and the A at 370 nm (A_{370}) was fixed at 0% as described previously (46). The RA curves thus obtained for different types of leucocytes of various vertebrates were compared.

RESULTS

I. Relative absorption (RA) curves of vertebrate leucocytes.

Mammalian leucocytes: The RA curves for human leucocytes (Fig. 1-a), and those of the cow (Fig. 1-b), pig (Fig. 1-c) and guinea pig (Fig. 1-d) were recorded. These curves for the leucocyte populations of vertebrates showed roughly two types of $A_{\max}$: one at a longer wavelength and the other at a shorter wavelength with a slight shoulder near 600 nm and a slight depression at 570 nm. Lymphocytes, monocytes and basophils gave the former type, and eosinophils and neutrophils the latter. Minor differences between the curves were also observed. The A at 600 nm (RA_{600}) was usually at a slightly higher wavelength than that of lymphocytes or monocytes from the same species, as shown in Fig. 1. In guinea pigs heterophilic granules, which are called neutrophils in man, are more con-

Fig.1-a.

Fig.1-b.

Fig.1-c.

FIG. 1-a. Relative absorption (RA) curves of human leucocytes. B: basophil, N: neutrophil (polymorph), E: eosinophil, L: lymphocyte, M: monocyte.

FIG. 1-b. RA curves of cow (*Bos taurus*, Gmelin) leucocytes.

FIG. 1-c. RA curves of pig (*Sus domesticus*, Brisson) leucocytes.

FIG. 1-d. RA curves of guinea pig (*Cavia cobaya*, Schreber) leucocytes. Symbols are the same as in Fig. 1-a.

spicuous than in man and they have sometimes been called pseudoeosinophils (4, 29). However, these heterophils were not examined here.

Fowl leucocytes: The RA curves of fowl leucocytes look similar to those of mammalian leucocytes, as shown in Fig. 2. But a special granular type has been identified as pseudoeosinophils. These are characterized by peculiar eosinophil ellipsoidal granules, as described by others (13, 23, 26). Their micro-structure has also been described (8, 38). The spectral profile of these cells resembled both the eosinophil and neutrophil types of mammalian leucocytes. The A_{max} of these eosinophils seemed to be at a shorter wavelength than those of mammalian

FIG. 2. RA curves of fowl (*Gallus domesticus*, Brisson) leucocytes. P: pseudoeosinophil. Other symbols are as in Fig. 1-a.

Fig. 3-a.

Gecko

Fig. 3-b.

Lizard

Fig. 3-c.

Snake

FIG. 3-a. RA curves of gecko (*Gekko japonicus*, Duméril et Bibon) leucocytes. N*: neutrophil, heterophil special (23), B: basophil, E: eosinophil, L: lymphocyte, M: monocyte

FIG. 3-b. RA curves of lizard (*Eumeces latiscutatus latiscutatus*, Hallowell) leucocytes.

FIG. 3-c. RA curves of snake (*Elaphe climacophora*, Bole) leucocytes. Symbols are the same as in Fig. 3-a.

eosinophils. Another kind of fowl leucocyte is the thrombocyte, and its RA curve resembles that of human monocyte rather than that of the fowl.

Reptilian leucocytes: RA curves were obtained for leucocytes from the gecko (Fig. 3-a), lizard (Fig. 3-b) and snake (Fig. 3-c). The leucocyte population of reptiles includes heterophil granulocytes. These cells are said to be similar to mammalian neutrophils and have been named variously as neutrophils by Alder-Huber (1), heterophil special granulocyte by Jordan (23) and amphochromatophils cited by Kumashiro (25). The terms used for the various leucocytes in Fig. 3 are those used by Jordan (23). The leucocyte populations of the gecko, lizard and snake had roughly similar RA curves. These curves resembled those of fowl leucocytes, but not those of mammalian leucocytes.

Amphibian leucocytes: RA curves are obtained for leucocytes of the tortoise (Fig. 4-a) and frog (Fig. 4-b). One type of leucocytes, heterophil neutrophils, looked different from typical eosinophils and neutrophils of mammals and have been named eosinophils with spheroidal or spindle-shaped granules by Alder-Huber

FIG. 4-a. RA curves of tortoise (*Clemmys japonica*, Temminck et Schlegel) leucocytes.

FIG. 4-b. RA curves of frog (*Rana nigromaculata nigromaculata*, Hollowell) leucocytes. Symbols are the same as in Fig. 3-a.

(1), pseudoeosinophils by Kyes (26) and neutrophils (heterophil) by Jordan (21). Another type did not resemble any known type of leucocyte but rather Spindelzellen (14) and these were excluded from the present study. Yet other leucocytes could not be typed and these are listed as unidentified leucocytes in the Tables. Despite these differences, the RA curves of amphibian leucocytes were very similar to those of reptilian leucocytes.

Fish leucocytes: The RA curves of leucocytes of two teleosts, the tuna (Fig. 5-a) and the eel (Fig. 5-b) were examined. The RA curves of almost all of these leucocytes had an A max at 524–516 nm. Thus unlike the leucocyte populations of other vertebrates they showed a very narrow range of RA curves. There is much confusion about suitable terminology for fish granulocytes, and names such as neutrophils by Meinertz (33), eosinophils by Drury (11), eosinophils (heterophil special) by Jordan (22, 23), amphochromatophils by Mori (36), pseudoeosinophils

FIG. 5-a. RA curves of tuna fish (*Thunnus orientalis*, Temminck et Schlegel) leucocytes.

FIG. 5-b. RA curves of eel (*Anguilla japonica*, Temminck et Schlegel) leucocytes. B: basophil, N*: neutrophil, heterophil special (23), E: eosinophil, E*: eosinophil, heterophil special (23), L: lymphocyte, L*: lymphoid (23), M: monocyte, T: thrombocyte, X: not identified

and amphochromatophils by Kumashiro (25) have been used. So, we were reluctantly obliged to use the terms used by Jordan (23).

II. Differences in the absorption parameters of vertebrate leucocytes.

The absorption maxima (A max) of the RA curves of vertebrates leucocytes are widely distributed between 550 nm and 510 nm (Figs. 1–5). As described previously (46), the RA spectra were classified into several types on the basis of the position of their A max: 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 1. All the A max values of vertebrates leucocytes were within these ranges. Human leucocytes and those of the cow, pig and guinea pig were classified as types I, II and III, fowl leucocytes as types I, II, III and IV, and reptilian and amphibian leucocytes as types II, III and IV. In contrast, the leucocytes of the tuna fish were all classified as type III (with the possible exception of unidentified cells) and those of the eel as types III and IV, as shown in Table 2. Thus, the A max range seems useful as a criterion for classification of the leucocyte types of vertebrates.

Absorption at above 550 nm in the RA curves indicates transmittance on the blue side due to formation of a basic dye complex in Wright solution. So that, the RA at 600 nm (RA 600) may be regarded as a parameter of blue light transmittance, and reflect the extent of basophilia of stained cells (46). Expressed as a percentage value, the RA 600 of human leucocytes ranged from 35% to 72%, and as expected, that of basophils was over 50% and that of eosinophils was less than 49%, as shown in Table 3. Similar differences between the RA 600 values of basophils and eosinophils were detectable in the leucocyte populations of the cow, pig, guinea pig and fowl, as well as in those of reptiles, amphibians and fishes, as shown in Table 4. In lower vertebrates, such as the fowl, frog, tuna and eel, ranges of the RA 600 were lower than these of mammalian leucocytes, but these results show that the RA 600 can be used as a parameter of basophilia of vertebrate leucocytes.

TABLE 1. *Cytospectral types of leucocytes.*

A max (nm): wavelength (nm) of maximum absorption

RA 460 (%): absorption at 460 nm as a percentage of the maximum absorption, RA 600 (%): absorption at 600 nm as a percentage of the maximum absorption

spectral type	Amax (nm)	RA 460 (%)	RA 600 (%)
I	550–536		> 50
II-a	535–525		> 50
-b	535–525		< 49
III-A	524–516	> 30	> 50
-a	524–516	< 29	> 50
-b	524–516	< 29	< 49
IV	515–510		

TABLE 2. *Correlations between spectral types and cell types of vertebrate leucocytes.*
B: basophil, N: neutrophil, E: eosinophil, L: lymphocyte, M: monocyte,
P: pseudoeosinophil, T: thrombocyte, N*: neutrophil, heterophil special (23),
E*: eosinophil, heterophil special (23), L*: lymphoid (23), X: not identified

Animal	Individual no.	Spectral types						
		I	II-a	II-b	III-A	III-a	III-b	IV
Man	1	B	LM				NE	
	2	B	LM			N	NE	
	3	B	LM			M	NE	
	4	B	L			M	NE	
	5	B	LM			N	NE	
	6	B	L			M	NE	
Cow	1	BL	LM			NE	E	
	2	BL	LM			NE		
	3	B	LM			N	NE	
Pig	1	B	LM			N	E	
	2	B	LM			N	E	
	3	BL	LM			N	E	
	4	BL	LM			NE	E	
Guinea pig	1		BLM			N	E	
	2	L	BLM			NE	NE	
	3		BLM			N	E	
	4		BLM			N	E	
Fowl	1	B	L		T		MP	E
	2	B	L		T		MP	E
	3	B		L	T		MP	E
Gecko	1		B		T	L	M	N*E
	2		B		T	LX	M	N*E
	3		B X		T	LX	M	N*E
Lizard	1		BL	B	T		M	N*E
	2		BL		T		M	N*E
Snake	1		BL		T	M	N*X	E
	2		L	B	T	M	N*X	E
	3		L	B	T	M	N*X	E
Frog	1		LM		T	B X		E
	2		L		T	BMX	N*	E
	3		BLM		T	X	N*	E
Tortoise	1		BL	L	T		N*	
	2		B M	L	T		MN*X M	E N*E
Tuna	1				T	BLMXN*	E	
	2				T	BLMX	N*E	
Eel	1					TBL * X	M	N*E*
	2					T L *	TM	N*E*

TABLE 3. *Distribution of RA 600 in leucocyte populations of mammals and the fowl.*

B: basophil, N: neutrophil, E: eosinophil, L: lymphocyte, M: monocyte,
P: pseudoeosinophil

The RA 600 can also be used to classify spectral types of leucocytes: namely, $RA\ 600 > 50\%$ (type a) and $RA\ 600 < 49\%$ (type b). According to this classification most basophils are of type a, whereas eosinophils are of type b. Mammalian blood contains blood platelets formed from megakaryocytes in the bone marrow, whereas the peripheral blood of animals lower than birds contain thrombocytes instead. The thrombocytes of lower vertebrates may be confused with Spindelzellen (14) which were excluded from this study. Most of the thrombocytes of lower vertebrates examined had an A max of type III, and RA 600 ranged of type a, as shown in Table 5. Type III seems to be the most widely distributed because it includes three or more leucocyte types as well as the thrombocytes of lower vertebrates shown in Fig. 4.

The RA at 460 nm (RA 460) seems to be higher in the RA curves of throm-

TABLE 4. *Distribution of RA 600 in leucocyte populations poikilothermal vertebrates.*
B: basophil, E: eosinophil, E*: eosinophil, heterophil special (23),
N*: neutrophil, heterophil special (23), L: lymphocyte, L*: lymphoid (23),
M: monocyte, T: thrombocyte, X: not identified

TABLE 5. *Distribution of RA 600 in thrombocytes of vertebrates.*

bocytes. The RA 460 values of different leucocyte types are compared in Table 6. Thrombocytes showed higher values ($>30\%$) of the RA 460 than other types of leucocytes, except for the eel. This, and the high RA 600 value distinguish thrombocytes from the other leucocyte types.

III. *Relations between spectral types and cell types of vertebrate leucocyte populations.*

As shown in Table 1, the RA spectra of vertebrate leucocytes were classified into 7 types on the basis of their A max, RA 600 and RA 460: type I, A max at 550–536 nm, RA 600 $>50\%$; type II-a, A max at 535–525 nm, RA 600 $>50\%$; type II-b, A max at 535–525 nm, RA 600 $<49\%$; type III-A, A max at 524–516 nm, RA 600 $>50\%$, RA 460 $>30\%$; type III-a, A max at 524–516 nm, RA 600 $>50\%$, RA 460 $<29\%$; type III-b, A max at 524–516 nm, RA 600 $<49\%$, RA 460 $<29\%$; type IV, A max at 515–510 nm. The correlation between the spectral types and cell types, determined cytologically using Wright's method, is shown in Table 2. There was a good correlation between spectral types and cell types in similar animal species, but not in very different animal species.

Most mammalian basophils were of type I, although those of the guinea pig were of type II-a. Eosinophils were mainly of type III-b but a few were of type III-a. The spectral types of the leucocyte populations of lower vertebrates were different. In the fowl, pseudo eosinophils, which are thought to be different from mammalian eosinophils (13, 23, 26) were of type III-b, while eosinophils were of type IV. Moreover in lower vertebrates, basophils were mostly of type II-a, though a few in the lizard and snake were of type II-b, whereas eosinophils were mostly of type IV. Thus, the spectral types of the granulocytes of lower vertebrates varied. In contrast, the thrombocytes of most of the species examined were consistently of type III-A. Those of the eel were of type III-a, but this is very similar to type III-A.

It is very difficult to identify all the leucocyte types in the blood of lower vertebrates, because previous reports on this subject are confusing and leucocytes of different cell species and animal species cannot all be identical (3, 44). The cells which could not be definitely identified are designated by the symbol X in Table 2. It can be seen that their spectral types were so widely distributed between types II and III that these cells cannot be regarded as a single cell species.

TABLE 6. *Distribution of RA 460 in vertebrate leucocytes of 'type III'.*

B: basophil, N: neutrophil, E: eosinophil, N*: neutrophil, heterophil special (23), E*: eosinophil, heterophil special (23), L: lymphocyte, L*: lymphoid (23), M: monocyte, T: thrombocyte, X: not identified

DISCUSSIONS

The Wright method (45) in combination with our method for cytospectral analysis (46) was adopted as being most suitable for the present study. To compare the absorption spectra of different leucocyte types it was necessary to adopt a constant way of expressing the spectra. For this purpose the RA curve in which the A max is fixed at 100% and the A 370 at 0% was employed. This RA curve proved very suitable in the present study, even for the leucocytes of lower vertebrates. Using the routine method for blood staining, it does not seem possible to determine leucocyte types in the blood of lower vertebrates. So we attempted to identify the leucocytes of lower vertebrates which differed from well known types in mammals such as basophils, neutrophils, eosinophils, lymphocytes and monocytes. The pseudo eosinophils of the fowl were easily distinguished from so-called heterophil granulocytes (4, 13, 23, 26, 28, 36). In most reports the term special heterophils has been adopted for granulocytes which differ from typical mammalian neutrophils (23). In the blood of the rabbit and guinea pig, special leucocytes have been observed in which heterophil cytoplasmic granules are more conspicuous than in leucocytes of man. These cells have sometimes been called pseudo eosinophils (4). In fowls, pseudo eosinophils were differed from genuine eosinophils, but they resembled human eosinophils in their cytospectral characters. In previous reports on the identification of leucocytes in lower vertebrates such as reptiles (1, 23, 25), amphibians (1, 3, 5, 14, 21, 36, 39, 44) and fishes (7, 11, 12, 22, 25, 33, 40, 44), various terms, such as Spezialleukozyten (30), neutraphils, heterophil special (23), bichromatophils cited by Kumashiro (25) and amphochromatophils cited by Mori (36) have been used and there is much confusion about the identification of leucocyte types. Thus in this work some leucocytes could not be identified and are shown by the symbol X in Table 2, although most were identified with those described as basophils, neutrophils, eosinophils, neutrophils (heterophil) or eosinophils (heterophil) by Jordan (23).

In vertebrates lower than the fowl, most eosinophil types were classified entirely as type IV. The only exceptions were the eosinophil type (identical with heterophil eosinophils of Jordan, 22) of fishes. A similar fixed spectral type was observed among the thrombocytes of lower vertebrates which was mostly classified as type III-A. There was a marked correlation between the spectral types of thrombocytes of various vertebrates and their cell types determined in the standard way. However, it is still difficult to distinguish so-called Spindelzellen (14) from thrombocytes. In this study we distinguished them from typical thrombocytes by the azurophil granulation of the latter (18, 22, 41) and we did not examine their RA spectra. Reptilian basophils showed RA spectra of types II-a and II-b, and those of amphibians of types II-a and III-a, whereas those of fish were all of type III-a. Thus the basophils of lower vertebrates can be divided into at least two types on the basis of their RA spectra. Moreover, reptilian basophils seem to be of type II, fish basophils of type III-a and amphibian basophils a mixture of types II-a and III-a. However, here is no strict distinction of so-called mast cell types in the fowl (43), guinea pig (10) or turtle (19, 23). Moreover, recent studies on granulocytes suggests a heterogeneous distribution of different granule types, such as azurophil and specific granules in neutrophils (2). Detailed cytological identi-

fication of the cells has not yet been fully correlated with the cytospectral characters of these cells.

The spectral types of agranulocytes were rather variable. For example, the monocyte types of amphibians were distributed widely in types II-a, III-a and III-b. Thus, agranulocytes do not seem to have a fixed spectral type. Most types of human leucocytes showed the same cytospectral characters as the corresponding types in other mammals, but not in lower vertebrates. The spectral types of eosinophils, including heterophil eosinophils of lower vertebrates were very similar, but those of other leucocytes were variable. The spectral types of leucocytes of lower vertebrates seemed to be much more restricted than those of higher vertebrates. In a single animal species it is common that basophils appeared bluer and less red than eosinophils. The peripheral blood of lower vertebrates, such as amphibians and fishes, contained cells which appeared from their morphological appearance to be immature (9, 19, 41). They were basophilic. We could not identify these cells and designated them by the symbol X in Tables 2, 4 and 6. Instead of this type, fish seemed to have leucocytes with a high RA 600 value. This means that the cytospectrum of leucocytes in the blood of lower vertebrates has a more basophilic profile, supporting the possibility of the presence of immature cells in the peripheral blood.

The present work shows that vertebrate leucocytes can be divided into several cytospectral types and that mammalian leucocytes are distinguishable from those of lower vertebrates on the basis of their cytospectra.

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