Characteristics of mouse leukocyte preparations before and after separation of cell types in small volumes of peripheral blood*

	Leukocytes (10^3 cells/mm^3)	Cell types (% total leukocyte count) Lymphocytes	Monocytes	Granulocytes
Before separation	9.3 (5.9–12.0)	85.8 (80.0–91.3)	3.7 (1.3–5.6)	10.5 (6.2–15.5)
After separation**	3.2 (1.9–4.3)	94.9 (92.3–97.2)	2.3 (1.5–3.5)	2.8 (1.1–4.6)
Recovery (%)	34.4 (26.0–46.0)	38.1 (27.6–52.9)	21.4 (12.5–28.4)	9.2 (4.3–13.4)

* Each value represents the mean obtained from 18 mice with ranges in parentheses. ** Contamination with erythrocytes and platelets was negligible in most samples.

Pasteur pipette over 0.6 ml of Lymphoprep (Accurate Chemical & Scientific Corporation, Hicksville, New York) in a 12×75 mm culture tube. The tube was centrifuged (international clinical centrifuge with a horizontal head) at 100× g for 25 min at 20 °C. Then, the upper layer of plasma and platelets was siphoned off and discarded by use of a Pasteur pipette, and the layer of enriched lymphocytes at the interface was collected carefully with another Pasteur pipette. The cells were transferred to a clean tube and, after the addition of 6–10 volumes of cold H-BSS, the tube was centrifuged at 160× g for 5 min. Subsequently, the supernatant was discarded, and the pellet of cells resuspended in 0.4 ml of H-BSS.

Results and discussion. The results are summarized in the table. It can be seen that 26–46% of the total leukocytes in the 18 blood samples were recovered after separation, and that the mean percent of lymphocytes was increased from 85.8 to 94.9% of the total cell count. Thus, the average number of lymphocytes obtained from 0.4 ml of blood was 38%. This level of recovery proved sufficient for selected biochemical studies.

The recovery of monocytes in the final suspensions (21%) was higher than expected. Presumably, it has its basis in the small volumes of blood which were employed and in the tendency of these cells to follow the lymphocytes during separation. Finally, the marked reduction in granulocytes from 11 to 3% of the total cell count (9% recovery) was considered as additional evidence for the ability to separate cell types by this method.

It should be noted that variations in the method with respect to the dimensions of the tube, the dilution of the blood, the volume of Lymphoprep, the period of centrifugation and the relative centrifugal force were found to have an adverse effect on the composition of the lymphocyte preparations. However, the care in collection of cells at the interface proved the most important variable since the removal of excess fluid was accompanied by the highest levels of contamination with erythrocytes and granulocytes.

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Autoradiography and differential hemoglobin staining as aids to the study of fish hematology

C.L. Mahajan and J.M.S. Dheer[1]

Department of Zoology, University of Rajasthan, Jaipur-302004 (India), 7 August 1978

Summary. The confused state of the existing knowledge regarding the cell types in peripheral blood and hematopoiesis of fishes has been highlighted. Inadequacies of techniques presently used have been pointed out and the advantages of using Graham-Knoll's method for haemoglobin staining when counter stained with Giemsa together with autoradiography using tritiated thymidine have been demonstrated.

Jakowska[2] commenting on the state of our knowledge of the hematology of fishes stated: '... The spirit which till now dominates the literature on the hematology of fishes seems to lack soundness. Several authors have expressed this very opinion on the confused state, the contradictory opinions, the inadequate descriptions, the unsatisfactory illustrations and the disagreements which arise from a highly varied nomenclature ... This paper has been prepared with the hope of helping newcomers to the study of hematology of fishes and to put an end to the "confused state" which still obtains in this branch of biology ...'

Inspite of a commendable effort made by her in recording original observations on a number of freshwater and marine fishes, and reviewing the existing literature with the stated end in view, the situation even after 2 decades of unprecedented technological progress since the publication of the above report, does not seem to have improved in any appreciable manner even though there has been considerable accretion to literature. For example, more than 900 references have been listed up to the end of the last decade[3] and a few hundreds would have been added in the seventies. That the 'confused state' continues is testified by a statement recently in a review[4] on the leucocytes of fish that '... The state of the literature concerning the eosinophils, and mast cells in fish is so confused that an entirely new approach to a study of these cells is warranted ...'

Part of the 'confused state' referred to above is due to the fact that, despite the ever-growing interest in fish blood and consequential number of research publications, very few workers have thought of using improved techniques. An example of the confusion is illustrated by the observation[5] that 'the nucleus of thrombocytes resembled the erythrocyte nucleus and the cytoplasm took on the same shade of pink as that of erythrocytes suggesting that the thrombocytes may also contain hemoglobin ...'. To our knowledge no one so far has unambiguously contradicted the state-

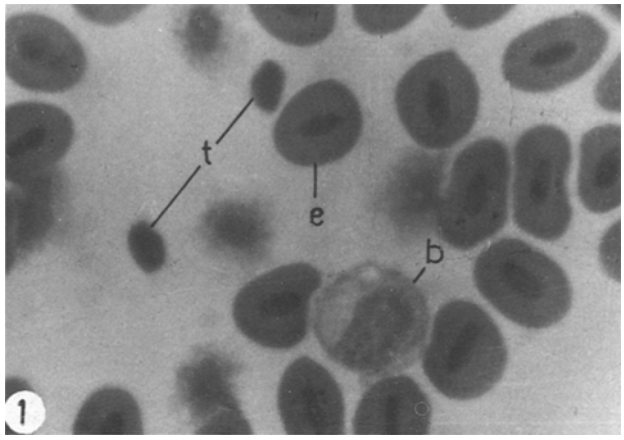

Fig. 1. Photomicrograph of a blood smear of *Channa punctatus* Bloch differentially stained with Graham-Knoll's method for hemoglobin and counterstained with Giemsa. e, erythrocyte; t, thrombocyte; b, basophil. × 950.

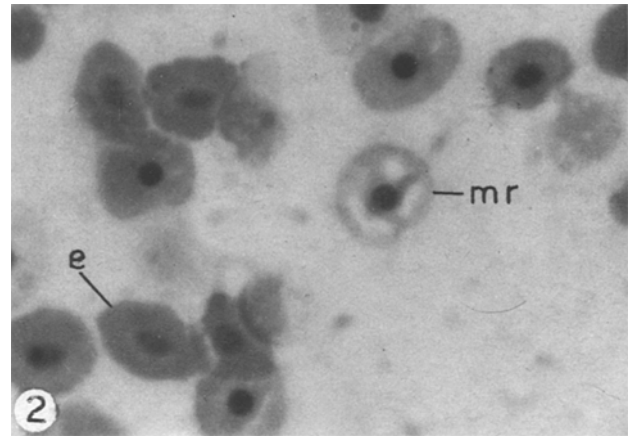

Fig. 2. Photomicrograph of head kidney imprint of *Channa punctatus* Bloch stained as in figure 1. mr, mature reticulocyte; e, erythrocyte. × 950.

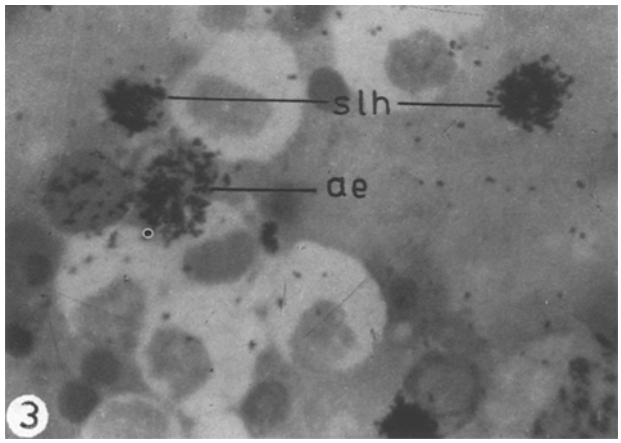

Fig. 3. Photomicrograph of spleen imprint of *Channa punctatus* Bloch labelled with tritiated thymidine and stained as in figures 1 and 2. slh, small lymphoid hemoblast; ae, acidophilic erythroblast. × 950.

ment inspite of the development and ever greater use of new techniques such as electron microscopy, autoradiography, fluorescence microscopy, etc.

As a preliminary to our effort in this laboratory to study and evaluate the effects of various environmental, pollutional, nutritional and other physiological stresses on fish by using blood as a sensitive indicator as a clinician does, we found ourselves confronted with this 'confused state'. Consequently to have dependable base line data, we tried to improve upon the techniques so far used in fishes to find a method clearly and unambiguously to distinguish the blood cells from each other and from rest of the cellular population both in the peripheral blood and the hematopoietic tissue.

To overcome the difficulty of clearly differentiating red blood cells from the thrombocytes due to the apparent nuclear similarity, and to verify the existence or otherwise of hemoglobin in the cytoplasm of the latter, to clear doubts created by the statement referred to above[5], it was thought essential to find a suitable stain which will differentially stain hemoglobin. After trials with techniques used for human blood[6-8], it was found that Graham Knoll's technique[6] gave the best results, particularly when counterstained with Giemsa in clearly and unambiguously identifying different cell types (figure 1). This combination of staining techniques has been successfully tried on the blood of a number of fish species belonging to the order Cypriniformes, Ophiocephaliformes and Clupiformes in this laboratory, with satisfactory results both on the peripheral blood and the hematopoietic tissues. It was particularly useful in identifying the various developmental stages during erythropoiesis because of the possibility with this technique of identifying hemoglobin in cytoplasm as soon as it begins to be synthesized in the developing erythrocytes (figure 2, mr).

Further to differentiate clearly the stem cells; it is essential to check the DNA synthetic activity. This could best be done by using autoradiography with tritiated thymidine administered i.m. as tracer 1.0 μCi/g b.wt. For suitable development of grains, 5 weeks exposure of blood smears for imprints was found suitable. Excellent results could be obtained when the autoradiographic imprints were differentially stained with hemoglobin and counterstained with Giemsa, as is evident from the accompanying photograph showing 2 of the developing stages synthesizing DNA (slh and ae, figure 3).

We have been using these methods for basic hematological studies[9], as well as to evaluate the effect of various environmental, pollutional or physiological stresses[10] on fish blood, for the last few years in this laboratory with consistently good results. It is hoped that the present report will stimulate others to use these results and to suggest further improvements.

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