

## MACROMOLECULAR SYNTHESIS IN DOGFISH PERIPHERAL BLOOD CELLS

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We have previously reported that tissue cultures of dogfish peripheral blood contain DNA-synthesizing cells without the addition of phytohemagglutinin (1, 2). The present cytochemical study concerns the possibility that such cells are constituents of dogfish blood in vivo, in contrast to the higher vertebrate classes in which DNA synthesis is not normally detected in the circulation.

### MATERIALS AND METHODS

Smooth dogfish (*Mustelus canis*) were obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, Mass. Adult, female animals, weighing 2.0–3.5 kg, were kept in tanks of running sea water at 15°C; peripheral blood was obtained from the caudal vein with vacutainers (Becton-Dickinson Co., Rutherford, N.J.) rinsed with 0.1 M sodium citrate prior to venipuncture.

Tritiated thymidine (TdR-<sup>3</sup>H), cytidine (CR-<sup>3</sup>H), and leucine (specific activities 1.9–2.0 Ci/mmole; Schwarz BioResearch Inc., Orangeburg, N.Y.) were administered by caudal vein injection at doses of approximately 0.5 μCi/gram body weight. Alternatively, 10-ml aliquots of freshly drawn whole blood were incubated at 18°C in closed sterile tubes containing the desired precursor (2 or 5 μCi/ml) and 0.1 ml of 0.1 M sodium citrate. The two labeling methods produced identical radioautographic results. Following exposure to isotope, the blood was centrifuged at 1500 g for 5 min and separate cytological preparations were made from the tightly packed disc of white cells and the underlying red cell pellet. The

cells were washed twice in cold elasmobranch Ringer's solution (299 mM NaCl, 12 mM KCl, 4.5 mM NaHCO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 5.5 mM glucose, and 360 mM urea, pH 7.8) and resuspended in 5 ml of fixative (absolute ethanol–acetic acid, 3:1). After 15 min at room temperature the cells were pelleted, resuspended in 0.1 ml of fixative, and a few drops of the suspension were allowed to dry on slides. Slides of cells labeled with tritiated nucleosides were then treated with 2.5% perchloric acid for 1 hr (4°C), while those exposed to leucine were extracted in two 15-min changes of cold 5% trichloroacetic acid (TCA). To assess the specificities of TdR-<sup>3</sup>H and CR-<sup>3</sup>H as precursors for DNA and RNA respectively, some slides were treated with DNase or RNase (Worthington Biochemical Corp., Freehold, N.J.) as described previously (3), followed by extraction for 12 hr in cold 5% TCA. Slides were dipped in Kodak NTB<sub>3</sub> liquid emulsion (diluted 1:1 with distilled water), exposed at 4°C, developed in Kodak D-19, and stained through the emulsion with Giemsa stain at pH 6.4. The percentages of labeled cells were determined by scoring about 200 of each type, while 25 were analyzed to obtain a mean grain count.

For cytophotometry, cells were fixed as above and stained by the Feulgen reaction with hydrolysis in 5 N HCl at 22°C (4, 4a). All staining was confined to nuclei and was absent from unhydrolyzed or DNase-treated preparations. Feulgen-DNA contents of individual nuclei were measured at 570 mμ by the one-wavelength, two-area procedure described by Garcia (5) by using a cytophotometer based upon the design of Pollister and Moses (6). 100 measurements

of each cell type were made per slide. After the Feulgen-DNA contents were plotted as frequency histograms, values belonging to the major diploid class were averaged to obtain the values of 2C given in the figures.

Samples of whole blood were prepared for electron microscopy as described previously (7).

## RESULTS

### DNA Synthesis

After a 5 min pulse with tritiated thymidine, only lymphocytes and erythroblasts were labeled, 4 and 15% respectively (Table I); as described previously, these are the two cells which synthesize DNA *in vitro* (1, 2). When the TdR-<sup>3</sup>H labeling was extended to 1 hr no additional cell types showed incorporation, although the percentages of labeled lymphocytes and erythroblasts increased to 20 and 39%, respectively, indicating that additional cells moved into the S phase during the hour. All labeling was confined to nuclei and was completely eliminated by DNase treatment.

Feulgen cytophotometry revealed that about 3% of the lymphocytes (Fig. 1) and 15% of the erythroblasts (Fig. 2) had nuclei with DNA contents in excess of 2C. These data are in agreement with the percentages of labeled nuclei and confirm that the TdR-<sup>3</sup>H incorporation manifests chromosome replication rather than DNA turnover. We have also observed mitotic figures in peripheral blood smears and, although it has been difficult to conclusively identify the specific cell types, we assume that they were derived from the DNA-synthesizing populations of lymphocytes and erythroblasts. Fig. 3 is the distribution of Feulgen-DNA contents among the mature erythrocytes. This population contains no cells with DNA contents greater than 2C, confirming its lack of TdR-<sup>3</sup>H labeling (Table I) and reinforcing the interpreta-

TABLE I  
DNA Synthesis in Dogfish Peripheral Blood Cells

Cell type	% labeled
Lymphocytes	4
Granulocytes	0
Thrombocytes	0
Erythroblasts	15
Mature erythrocytes	0

Whole blood was incubated for 5 min with thymidine-<sup>3</sup>H, 2  $\mu$ Ci/ml, as described in text. Radioautographs exposed for 11 days.

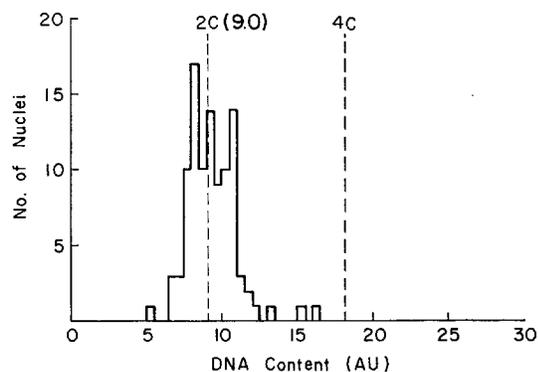


FIGURE 1 Distribution of Feulgen-DNA contents among 100 dogfish peripheral lymphocytes.

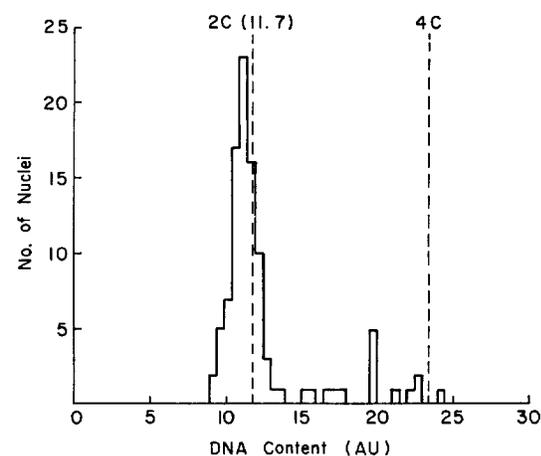


FIGURE 2 Feulgen-DNA contents of peripheral erythroblasts (population measured consisted of 100 mid- or late-polychromatic cells).

tion that only DNA-synthesizing cells display increased Feulgen dye contents. Erythrocyte nuclei with less than the 2C amount were occasionally encountered, but they occurred only in degenerating cells. It has been our impression that karyolysis accompanies erythrocyte degeneration, with no evidence of nuclear extrusion.

The cytophotometry data reveal a highly significant difference between the 2C values for lymphocytes (9.0) and erythroblasts (11.7). A difference of comparable magnitude, and significance, was noted among measurements from a second animal. Mammalian small lymphocytes have about 10% less Feulgen-DNA than other diploid cells (8-11), while the dogfish lymphocyte

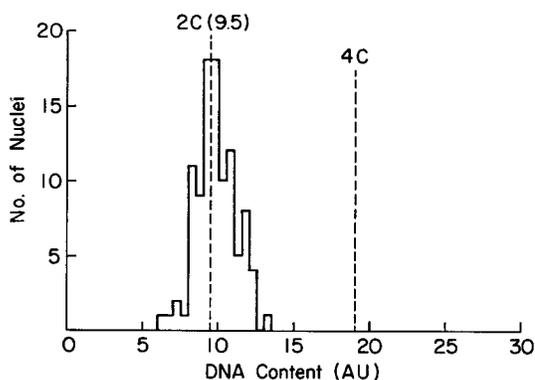


FIGURE 3 Feulgen-DNA contents of dogfish mature erythrocytes.

contains about 25% less than the erythroblast. Differences of this sort are probably apparent ones, related to the degree of DNA-protein compaction in nuclei of different volumes. This is supported by recent findings of Garcia (12) who detected increases in Feulgen-DNA of leukocytes after expanding their nuclei in hypotonic solutions. The dogfish lymphocyte has a nucleus sufficiently smaller than the erythroblast's to account for the observed difference in DNA contents. Further, the mature erythrocytes (Fig. 3) have nuclei approximately the same size as the nuclei of lymphocytes (Fig. 1) and also have a similar value of 2C, 9.5 vs. 9.0 respectively.

#### RNA and Protein Synthesis

When samples of peripheral blood were incubated for 1 hr with cytidine-<sup>3</sup>H or leucine, the only cells which showed incorporation were again lymphocytes and erythroblasts (Table II). The RNA- and protein-synthesizing erythroblast populations were about three times larger than the corresponding lymphocyte populations. Although the rates of RNA synthesis, reflected by the grain counts, were similar in the two cells, the level of protein synthesis in erythroblasts was twice that in lymphocytes. In addition, more erythroblasts synthesized protein (67%) than RNA (45%), suggesting the existence of stable messenger RNA in at least part of the population. This was confirmed by the results obtained when RNA synthesis was inhibited with actinomycin D (Table II). Lymphocytes exposed to the antibiotic for 5 min and then to either CR-<sup>3</sup>H or leucine-<sup>3</sup>H showed an 80% inhibition in both RNA and protein synthesis.

TABLE II

RNA and Protein Synthesis and the Effects of Actinomycin D

	RNA			Protein		
	% labeled	Grain count	Actino- mycin D	% labeled	Grain count	Actino- mycin D
Lymphocytes	17	32	7	20	11	2
Erythroblasts	45	40	6	67	24	18

Freshly drawn samples of blood were exposed for 1 hr to cytidine-<sup>3</sup>H or leucine-<sup>3</sup>H, 5  $\mu$ Ci/ml. Actinomycin D, 10  $\mu$ g/ml, was added to two other samples for 5 min prior to incubation with isotope as above. Radioautographic exposure: 20 days. Actinomycin D had no effect on the percentages of cells labeled.

However, while erythroblast RNA synthesis also declined 85%, protein synthesis displayed only a slight reduction, about 25%. This relative insensitivity of erythroblast protein synthesis to the antibiotic's virtually complete curtailment of RNA production suggests that reutilization of messenger RNA's, and perhaps ribosomes also, is more extensive in these cells than in lymphocytes. Stable messenger RNA is also a feature of erythrocyte development in amphibians (3), birds (13), and mammals (14, 15).

#### DISCUSSION

The peripheral blood of adult dogfish contains about  $4 \times 10^3$  red cells/ml and  $10^3$  white cells; lymphocytes comprise half of the white cell population, the balance consisting of thrombocytes, eosinophils, and developing erythroid cells, with polymorphonuclear leukocytes being absent (16). The circulating lymphocytes (Fig. 4) have the high nuclear:cytoplasmic ratio and indented nucleus which characterize the mammalian small lymphocyte. However, in contrast to their mammalian counterpart, the dogfish lymphocyte has relatively diffuse chromatin, resembling that seen in phytohemagglutinin-transformed human lymphocytes (17). The expanded chromatin of the dogfish lymphocyte is probably a manifestation of the nucleic acid synthesis here described. The erythroblasts (Fig. 5) have, relative to the lymphocyte, a decreased nuclear:cytoplasmic ratio, chromatin in a more condensed state, and a reduced cytoplasmic basophilia when stained with azure B. With acid dyes, such as fast green, the erythroblast popula-

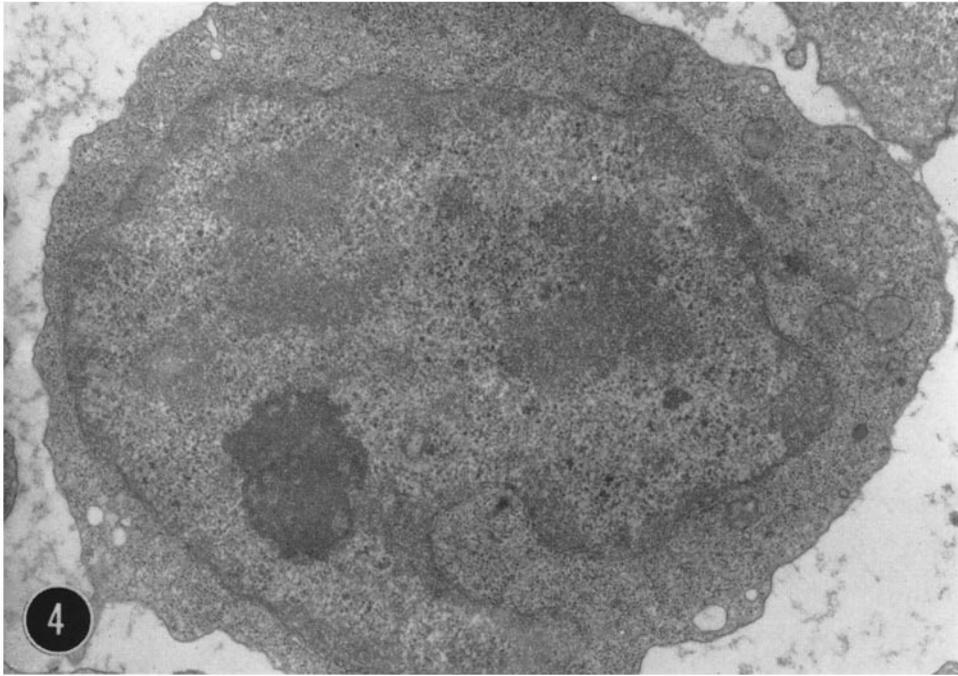


FIGURE 4 Peripheral lymphocyte.  $\times 14,000$ .

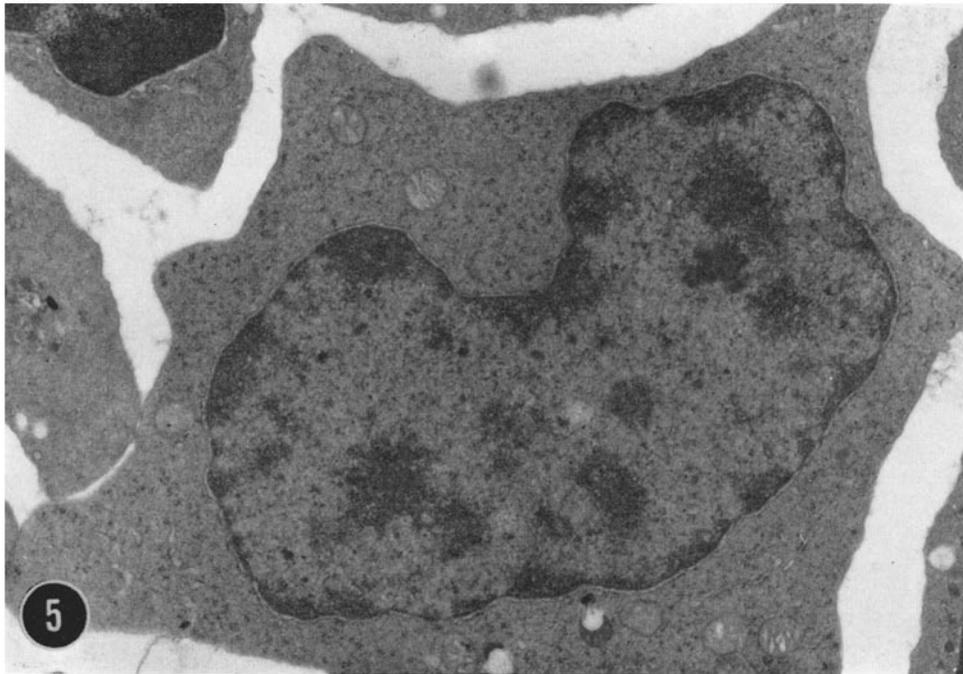


FIGURE 5 Peripheral erythroblast.  $\times 10,000$ .

tion displays a range of cytoplasmic staining intensities, presumably reflecting different amounts of hemoglobin. Although these transitional stages were not separately analyzed in this report, the data presented concern primarily mid- and late-polychromatic erythroblasts since these are by far the most prevalent stages.

Since the erythropoietic process involves both cell division and differentiation, it is not surprising that dogfish erythroblasts synthesize DNA. More interesting is their presence in the peripheral blood, since erythrocyte development takes place centrally in mammals. That the circulation can be considered part of the dogfish erythropoietic system is demonstrated also by the finding of increased numbers of erythropoietic cells in the circulation after withdrawing about 25% of the blood volume (18, and T. Pederson, unpublished data). Circulating erythropoietic cells have been described in amphibians by Grasso and Woodard (19). Although they employed splenectomy as a means to potentiate peripheral erythropoiesis in newts, some activity was also observed in unoperated animals. With respect to the maintenance of dogfish circulating lymphocytes, it had been thought previously that they arise solely by differentiation of lymphoid precursors in the spleen and kidneys (20). However, the present findings demonstrate a small population of lymphocytes that proliferate in the peripheral blood as well. Thus, with respect to the production of both erythrocytes and lymphocytes, the dogfish circulation can be viewed as a component of the over-all hematopoietic system.

#### SUMMARY

Radioautographic and cytophotometric studies of dogfish blood demonstrated that circulating lymphocytes and erythroblasts, but not other blood cells, synthesize DNA, RNA, and protein. Erythroblasts contain relatively stable messenger RNA as manifested by the insensitivity of their capacity for protein synthesis after RNA production is inhibited by actinomycin D. The results support the hypothesis that the peripheral circulation of the dogfish is a component of its hematopoietic system.

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