moving bands, which appear in chronic diseases as more frequent and much faster than in normal samples, remains unclear sofar. Their presence might be explained by the formation of oligomers or by a disbalance in the oxido-reductive status of the enzyme molecule.

A similar study as reported herein has been published recently[23].

Zusammenfassung. Es wird über elektrophoretische Untersuchungen zum mikrosomalen Enzym Hexose-6-Phosphat-Dehydrogenase (H6PD) aus menschlichen Leukocyten, Plazentagewebe und Leber bei Gesunden und Kranken berichtet. Ferner wird durch Analyse des Enzyms H6PD bei zwei verschiedenen Glukose-6-Phosphate-Dehydrogenase (G6PD) Phänotypen gezeigt, dass die beiden Enzyme H6PD und G6PD beim Menschen nicht gemeinsamer genetischer Kontrolle unterliegen.

K. G. BLUME, G. M. SCHMIDT, H. H. HEISSMEIER and G. W. LÖHR

Medizinische Universitätsklinik, Hugstetter Strasse 55, D-78 Freiburg im Breisgau (German Federal Republic, BRD), 5 December 1974).

[23] L. A. ROSENSZAJN and D. JOSEPH, Biochim. biophys. Acta *364*, 38 (1974).

Lymphocyte Transformation Studies of Sea Mammal Blood

A previous communication from this laboratory[1] described a technique for cytogenetic analysis of the dolphin (*Tursiops truncatus*). The following is a report of studies on the in vitro mitogen-induced blastogenic response of lymphocytes from Delphinidae. To our knowledge, these are the first in vitro cellular immune studies reported in sea mammals.

Materials and methods. A male and a female member of each of the species, *T. truncatus* (dolphin), *Orcinus orca* (killer whale), and 1 male *Globicephala melaena* (pilot whale) were studied. These aquatic mammals are captive specimens of an oceanarium collection (Sea-Arama of Texas, Galveston, Texas). Peripheral blood from each animal was drawn by venipuncture into a sterile heparinized (20 units/ml of blood) syringe. Lymphocyte cultures were established by a method similar to that used for the study of human peripheral blood lymphocytes[2]. Triplicate culture tubes containing 0.1 ml of heparinized whole peripheral blood in 3 ml of medium were innoculated with either 0.1 ml phosphate-buffered saline (PBS, control tubes), 0.1 ml of 1:2 dilution of phytohemagglutinin-M (PHA, Difco Laboratories, Detroit, Michigan), or 0.1 ml of pokeweed mitogen (PWM, Grand Island Biological Company, Grand Island, New York). The culture medium consisted of Earle's minimal essential medium (MEM) supplemented with 20% heat-inactivated fetal calf serum, 50 µg erythromycin, 200 µg streptomycin, and 1.5 µg sodium bicarbonate/ml. After 2 to 13 days of incubation at 37 °C and 5% CO_2, 0.5 µCi of tritiated thymidine were added to each culture tube. Approximately 24 h after labeling, the cells were washed twice with distilled water to lyse the erythrocytes, and the cell pellet was precipitated with 3 ml 5% cold trichloroacetic acid (TCA). The precipitate was then washed with methanol and digested with 0.2 ml of 0.1 *N* sodium hydroxide. 10 ml of scintillation cocktail consisting of 4 g of Butyl-PBD (Beckman Instruments, Fullerton, Calif.), 100 mg of POPOP (Packard Instrument Co., Downer's Grove, Ill.), and 100 ml Bio-Solv BBS-3 (Beckman)/1000 ml of scintillation grade toluene (Beckman) were added to each tube at least 2 h before counting in a Beckman LS-150 counter. The counts per minute (cpm) of triplicate tubes were averaged and the specific incorporation (SI) of tritiated thymidine was calculated by the formula SI = cpm/control cpm. In some experiments, additional culture tubes containing only blood and medium were set up for cell viability studies. On the day of harvest these cells were washed twice with PBS, and viability was determined by trypan blue exclusion. Additional studies were run to demonstrate that the specific incorporation results were actually due to thymidine incorporation in blastic and mitotic cells as had been found in other mammalian species tested by lymphocyte transformation. Autoradiographs of dolphin blood were done at 5 days on PWM stimulated cells at the same time as tubes were harvested for morphological counts of transformed cells and tubes for liquid scintillation counting. Culture of lymphocytes were labeled 24 h before harvesting.

Aliquots of the cell sediment from triplicate tubes were carefully smeared on hematology microscopic slides for staining (Wright's). Counts of transformed (lymphoblastic) and mitotic cells/1,000 cells were read in triplicate.

Radioactive labeled blood smears on glass microscope slides were immersed at 40 °C into a 50% solution of Kodak NTB-2 nuclear track emulsion and deionized distilled water. The slides were then room dried and stored in light-tight boxes for 40 days exposure to the radioactive label. They were then developed at 17 °C in a Dektol solution, fixed with Kodak acid fixer, and then washed in deionized water at 17 °C. After drying, the

Table I. Response of peripheral blood cultures from the killer whale (*O. orca*) to in vitro exposure to PHA

Day of assay	PBS (CPM)	PHA (CPM)	SI[a]	Cell viability (%)
3	77[b]	3179	40.7	83
	118[c]	4049	34.3	79
5	220	5011	22.8	72
	353	6864	19.4	76
7	138	4359	31.6	60
	412	4655	11.1	62
10	289	3306	11.4	36
	78	2086	26.7	40
14	330	395	1.2	30
	416	393	0.9	36

[a] SI, specific incorporation of tritiated thymidine = CPM PHA/CPM PBS (SI > 3.0 = positive). [b] Total leukocyte count = 5,600/mm³; differential = 32% mononuclear cells; 68% polymorphonuclear. [c] Total leukocyte count = 7,000/mm³; differential = 25% mononuclear cells; 75% polymorphonuclear.

[1] N. PRASAD, D. M. MUMFORD, P. B. BARSALES, T. WHITMAN and J. R. WILBUR, Experientia *26*, 1167 (1970).
[2] D. M. MUMFORD, P. B. BARSALES, C. E. FLOWERS, JR., J. R. WILBUR and R. H. EPPRIGHT, Fedn. Proc. *29*, 369 (1970).

Table II. In vitro response of pilot whale ((*G. melaena*) [a] lymphocytes to PHA

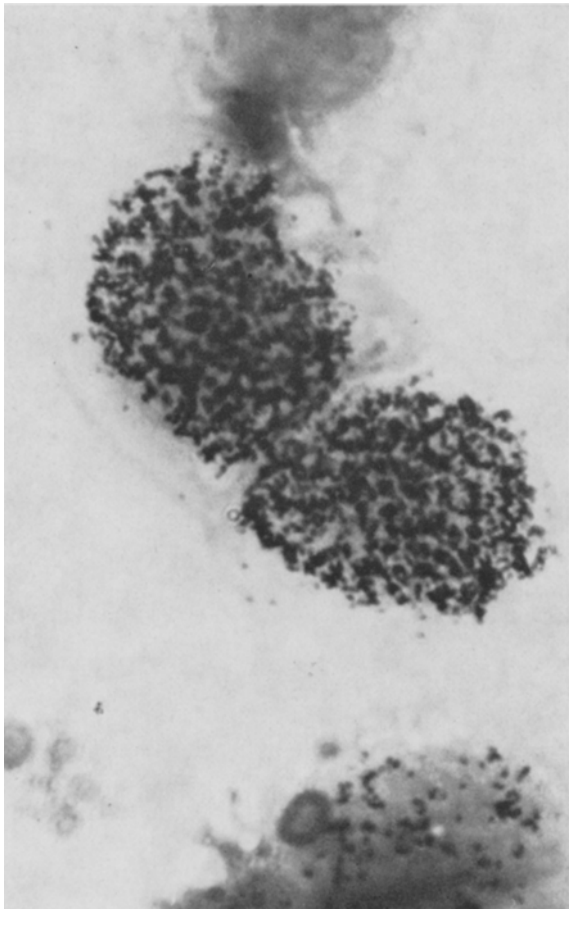
Day of assay	PBS (CPM)	PHA (CPM)	SI [b]	Cell viability (%)
3	155	259	1.7	87
5	431	495	1.1	71
7	229	853	3.7	55
10	131	137	1.0	44
14	152	127	0.8	35

[a] Total leukocyte count = 8,400/mm³; differential = 26% mononuclear cells; 74% polymorphonuclear. [b] SI > 3.0 = positive.

Table III. In vitro response of dolphin *T. truncatus* peripheral blood cultures to PHA and PWM.

	PBS (cpm)	PHA (cpm)	SI/PHA	PWM (cpm)	SI/PWM
a)	62	7,432	119.9	14,314	230.9
b)	85	1,236	14.5	4,349	51.2

a) Total leukocyte count = 13,200/mm³; differential = 30% mononuclear cells; 70% polymorphonuclear. b) Total leukocyte count = 12,000/m³; differential = 30% mononuclear cells; 70% polymorphonuclear.



Autoradiograph (40 days) of PWM stimulated dolphin lymphocyte (center). Note heavy nuclear grains and visible nucleoli.

prepared slides were then stained in a basic solution of Paragon multiple stain, examined and photographed with a Leitz Orthoplan microscope.

Results. Peripheral blood cultures from both the male and the female killer whales responded well to stimulation by PHA (Table I). A maximum response in terms of specific incorporation (SI) occurred after a 3 day culture period. In terms of cpm of tritiated thymidine, the response was maximum at day 5. However, the background incorporation of isotope (cpm PBS) was higher at this time also. Both cpm of PHA cultures and SI could be seen to decrease with time as the percentage of viable cells decreased. Peripheral blood cultures of the pilot whale (Table II) established under the same conditions and on the same day as the killer whale cultures made only a poor response to PHA (maximum SI of 3.7 on day 7).

In addition to PHA stimulation, dolphin blood samples were incubated with PWM (pokeweed mitogen). This substance may primarily stimulate the B cells of the immune compartment[3]. Peripheral blood cultures of both the male and female dolphin responded well to both PHA and PWM, with a maximum SI response to both mitogens on day 5 (Table III). The response of both dolphins to PWM greatly exceeded that of PHA. This is in contrast to our experience with human[4] and mouse[3] lymphocyte cultures, in which the SI and PHA cultures may be several times greater than that of PWM cultures.

Concomitant cultures of dolphin blood counted for transformation and mitotic indices revealed a 53–70% rate of stimulation in PWM studies. Autoradiographic analysis showed labeling of lymphoblastic cells with prominent nucleoli (Figure). Interestingly, a rare cell, thought to be of the erythroid series, was found with grain counts.

Discussion. Although studies on the immunoglobulins of aquatic mammals have been reported[5], we could find no information on the lymphocyte transformation response of these animals. Using a whole blood culture technique, similar to that used in our laboratory for the study of human lymphocyte transformation[2], we were able to show that in vitro cultures of lymphocytes from both the killer whale (*O. orca*) and the dolphin (*T. truncatus*) responded well to the mitogen PHA. PHA has been shown to specifically stimulate the thymus-dependent, or T cell, population of lymphocytes[6]. Although prepared the same day and under the same culture conditions, for reasons not easily apparent, lymphocytes from the pilot whale (*G. melaena*) responded only poorly to PHA. The reason for this difference is not obvious, since there was no difference in leukocyte viability between the pilot and the killer whales, and the total and differential leukocyte counts were similar.

Although a matter of current debate, the response to PWM (a mitogen which is thought to stimulate primarily thymus-independent, or B cells[4]) was studied only in the dolphin. Dolphin lymphocytes, in contrast to those of humans[4] and mice[3], were stimulated to a greater degree by PWM than by PHA. No ready explanation is available.

[3] G. D. Stockman, M. T. Gallagher, L. R. Heim, M. A. South and J. J. Trentin, Proc. exp. Soc. Biol. Med. *136*, 980 (1971).
[4] Unpublished results.
[5] D. R. Nash and J. P. Mach, J. Immun. *107*, 1424 (1971).
[6] G. E. Rodey and R. A. Good, Int. Arch. Allergy appl. Immun. *36*, 399 (1970).

Although on stained transformation smears and radiographic prints, a rare, labeled erythroid series cell was found, it is not thought that this event contributed significantly to the total cpm found in the PWM stimulated dolphin tubes. If a consistent finding, the differential PHA, PWM response deserves further investigation in greater numbers of sea mammals.

[7] We gratefully acknowledge the assistance of the personnel of the Sea-Arama of Texas, Galveston, especially Dr. KEN GRAY.
[8] This work was partially supported by the Rockefeller Foundation Grant No. 67050.

Zusammenfassung. Erster Nachweis der in vitro-Stimulation peripherer Blutlymphozyten bei Meersäugern durch Phytohämagglutinin und «pokeweed»-Mitogen.

D. M. MUMFORD, G. D. STOCKMAN, P. B. BARSALES, T WHITMAN and J. R. WILBUR[7,8]

Department of Obstetrics and Gynecology. Baylor College of Medicine, Texas Medical Center, Houston (Texas 77025, USA), and M. D. Anderson Hospital and Tumor Institute, Houston (Texas, USA), 28 October 1974.

Defective Ristocetin and Bovine Factor VIII-Induced Platelet Aggregation in Normal Rats

Recent work has indicated that high molecular weight factor VIII-related material (HMW-F-VIII) of different animal origin induces aggregation of human platelets[1–4]. The identity of HMW-F-VIII with von Willebrand factor has been suggested[2,3,5,6].

Platelets from patients with von Willebrand's disease are not aggregated by the antibiotic ristocetin, unless a source of human HMW-F-VIII is added[7–11]. In contrast, platelets from patients with Bernard-Soulier (hereditary giant platelet) syndrome are unresponsive to ristocetin even in the presence of HMW-F-VIII; in addition, they are not aggregated by bovine fibrinogen preparations[12–15].

Platelets from rats, in contrast to platelets from guinea-pigs, were reported[16] not to be aggregated by a bovine fibrinogen preparation (Kabi) lately shown to contain HMW-F-VIII as the actual aggregating stimulus[2]. The present study was undertaken to evaluate whether rat platelets are indeed unresponsive to purified bovine HMW-F-VIII and whether, in analogy with platelets from Bernard-Soulier patients, they are also refractory to ristocetin.

Blood was obtained by venipuncture from normal human voluteers and by intracardiac puncture from guinea-pigs and rats. One-tenth (v/v) 3.8% trisodium citrate was used as anticoagulant. 3 different strains of rats were used: Sprague Dawley (Charles River), Wistar (Morini) and Long Evans (Servier). The animals were anesthetized with ether just before blood collection. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described[17]. Platelets were separated from plasma either by gel filtration[18] or by a centrifugation-resuspension technique, repeated 4 times[19]. In each test, the platelet number was adjusted to about 300,000/µl.

Ristocetin A (lot 3) (kindly supplied by Lundbeck & Co., Copenhagen, Denmark), containing less than 8% ristocetin B, was dissolved in isotonic saline; adenosine-5′-diphosphate (Sigma; St. Louis, Missouri, USA) and Thrombofax (batch No. 8L 118, Ortho Diagnostic; Raritan, N.J., USA) were used as previously described[20]; native collagen fibrils from equine tendons were suspended in an organic isotonic buffer pH 2.8 (Hormon Chemie, Munich, Germany); 5,000 times purified HMW-F-VIII (from bovine plasma) was a gift from Drs. J. VERMYLEN and D. BOTTECCHIA, Laboratory of Blood Coagulation, University of Leuven, Belgium; partially purified HMW-F-VIII preparations were also obtained by gel chromatography of porcine or human PPP as previously described[2]. Human HMW-F-VIII was subsequently treated with neuraminidase, a procedure leading to the development of an aggregating activity towards human platelets[4].

Human, rat and guinea-pig platelets were similarly aggregated by ADP (up to 10^{-7} *M*) and Thrombofax (1/5 dilution); in contrast, 5 to 10 times more concentrated collagen suspensions were required to obtain similar changes in light transmission for rat as compared to both human and guinea-pig platelets, a finding already reported by CONSTANTINE[21]; neither ristocetin (up to 3 mg/ml) nor bovine, porcine or human neuraminidase-treated HMW-F-VIII preparations induced rat platelet aggregation (Figure 1); no consistent differences were observed among the 3 strains of rats used.

[1] C. D. FORBES and C. R. M. PRENTICE, Nature New Biol. *241*, 149 (1973).
[2] M. B. DONATI, G. DE GAETANO and J. VERMYLEN, Thromb. Res. *2*, 97 (1973).
[3] T. R. GRIGGS, H. A. COOPER, W. P. WEBSTER, R. H. WAGNER and K. M. BRINKHOUS, Proc. natn. Acad. Sci., USA *70*, 2814 (1973).
[4] J. VERMYLEN, M. B. DONATI, G. DE GAETANO and M. VERSTRAETE, Nature, Lond. *244*, 167 (1973).
[5] G. DE GAETANO, M. B. DONATI and J. VERMYLEN, Thromb. Diath. haemorrh., *32*, 549 (1974).
[6] T. R. GRIGGS, W. P. WEBSTER, H. A. COOPER, R. H. WAGNER and K. M. BRINKHOUS, Proc. natn. Acad. Sci., USA *71*, 2087 (1974).
[7] M. A. HOWARD and B. G. FIRKIN, Thromb. Diath. haemorrh. *26*, 362 (1971).
[8] M. A. HOWARD, R. J. SAWERS and B. G. FIRKIN, Blood *41*, 687 (1973).
[9] D. MEYER, C. S. P. JENKINS, M. DREYFUS and M. J. LARRIEU, Nature, Lond. *243*, 293 (1973).
[10] J. D. OLSON, D. N. FASS, E. J. W. BOWIE and K. G. MANN, Thromb. Res. *3*, 501 (1973).
[11] H. J. WEISS, J. ROGERS and H. BRAND, J. clin. Invest. *52*, 2697 (1973).
[12] T. C. BITHELL, S. J. PAREKH and R. R. STRONG, Ann. N.Y. Acad. Sci. *201*, 145 (1972).
[13] J. P. CAEN and S. LEVY-TOLEDANO, Nature New Biol. *244*, 159 (1973).
[14] J. P. CAEN, S. LEVY-TOLEDANO, Y. SULTAN and J. BERNARD, Nouv. Revue. fr. Hémat. *13*, 595 (1973).
[15] M. A. HOWARD, R. A. HUTTON and R. M. HARDISTY, Br. med. J. *2*, 586 (1973).
[16] Z. SINAKOS and J. P. CAEN, Thromb. Diath. haemorrh. *17*, 99 (1967).
[17] G. DE GAETANO, J. VERMYLEN and M. VERSTRAETE, Thromb. Diath. haemorrh. *24*, 419 (1970).
[18] O. TANGEN and J. BERMAN, *Advances in Experimental Medicine and Biology* (Eds. P. M. MANNUCCI and S. GORINI; Plenum Press, New York 1972), p. 235.
[19] J. VERMYLEN, M. B. DONATI and G. DE GAETANO, Acta med. scand. suppl. *525*, 19 (1971).
[20] G. DE GAETANO, A. VANDENBUSSCHE and J. VERMYLEN, Experientia *28*, 1127 (1972).
[21] J. W. CONSTANTINE, Nature, Lond. *214*, 1084 (1967).