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.

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

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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).

These results indicate that the previously reported absence of rat platelet aggregation by bovine fibrinogen[16], was in fact due to the lack of response of rat platelets to bovine HMW-F-VIII; this is reinforced by the observation that other preparations of HMW-F-VIII, capable of inducing human and guinea-pig platelet aggregation, failed to clump rat platelets.

Although ristocetin in high concentration is known to precipitate human fibrinogen[7], we were unable to detect any precipitate after addition of ristocetin (3 mg/ml) to rat PRP; however, at concentrations above 3 mg/ml, the addition of ristocetin to rat PRP provoked an immediate decrease of light transmission beyond the 0% value (PRP was calibrated at 15% transmission, taking the corresponding PPP as 100%); some granular material, resembling platelet aggregates, was visible, but was possibly due to fibrinogen precipitation; indeed no fibrinogen[22] could be detected in either PRP or PPP after addition of ristocetin and subsequent centrifugation at 3,000 *g* for 15 min.

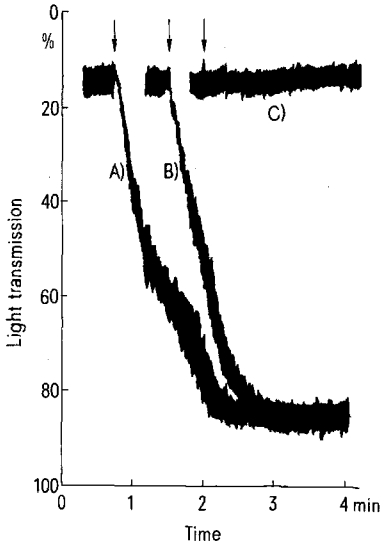

Fig. 1. In vitro platelet aggregation induced in guinea-pig PRP by bovine HMW-F-VIII (2 µg/ml) (curve A) and by ristocetin (1.5 mg/ml) (curve B). Absence of rat platelet aggregation by the above-mentioned substances (curve C). No shape change[24] was observed in any system. The tests were performed in a Born-Michal MK IV Aggregation and Shape Change Monitor (Pharmacological Research, England) connected to a two-channel chart recorder (Servoscribe 2, type RF 520, Smith's Industries Ltd; England).

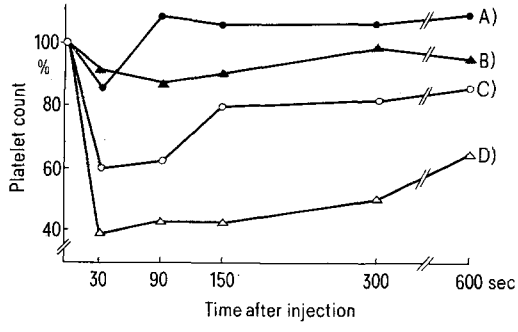

Fig. 2. In vivo platelet aggregation (expressed as percent of initial platelet count) induced in guinea-pig by i.v. injection of ristocetin (20 mg/kg body weight, curve C) or of bovine HMW-F-VIII (60 µg/kg body weight, curve D). Absence of rat platelet aggregation by the above-mentioned substances (curves A and B respectively). Each curve is the mean of 2 different experiments. The method of MacKenzie et al.[25] was followed for studies in guinea-pigs and the method of Kobayashi and Didisheim[26] for studies in rats.

Addition of either human or guinea-pig PPP to rat PRP before ristocetin did not provoke platelet aggregation. Both gel-filtered and extensively washed rat platelets (resuspended either in *tris* phosphate buffer pH 7.4 or in rat serum or in human PPP) were insensitive to all HMW-F-VIII preparations (at all concentrations tested) and to ristocetin (up to 3 mg/ml); the same platelet preparations, however, were aggregated by ristocetin at a concentration of 5 mg/ml; extensively washed human platelets, resuspended in rat PPP, were normally aggregated by ristocetin. Both observations clearly indicate that a plasmatic defect can be excluded in rat and that the refractoriness to ristocetin is due to rat platelets themselves.

Recent work[10] has shown that platelets obtained from bovine, porcine, canine and ursine species were not aggregated, in their own plasma, by ristocetin; a species specificity for this substance, at least in vitro, has been suggested; that the defective ristocetin-induced rat platelet aggregation would be due to some in vitro artifact, can be excluded by in vivo experiments showing that both bovine HMW-F-VIII and ristocetin induced a marked fall in platelet count in guinea-pigs, but were without effect in rats (Figure 2). Similar results have been obtained by F. DeClerck (personal communication) after infusion in guinea-pigs and rats of an HMW-F-VIII-rich bovine fibrinogen preparation.

The lack of response of rat platelets to heterologous HMW-F-VIII and to ristocetin seems not to be of importance for the haemostatic mechanism of this animal species, as none of the strains studied suffered from haemorrhagic diathesis; although the question whether rats need von Willebrand factor for normal haemostasis remains unanswered, our observations add further support to the hypothesis put forward by Constantine[21] that rat platelets are less sensitive than are platelets from other animal species, to stimuli which may be related to the in vivo formation of platelet thrombi in rat vessels. In addition, our results could be relevant for a better understanding of some human platelet disorders.

Indeed, absence of platelet aggregation by both ristocetin and bovine fibrinogen preparations (the latter being rich in HMW-F-VIII) has recently been reported[13] in some patients affected with Bernard-Soulier syndrome; it has been suggested[13] that Bernard-Soulier platelets, possibly due to a reduced amount of sialic acid on their membrane[23], are lacking a receptor for ristocetin of for a ristocetin-HMW-F-VIII (von Willebrand factor) complex.

Since patients affected with Bernard-Soulier syndrome are relatively rare, rat platelets could be used as a model for future investigations on the glycoprotein composition of the platelet membrane possibly involved in bovine HMW-F-VIII- and ristocetin-induced aggregation of human platelets[4].

[22] C. Vermylen, R. A. De Vreker and M. Verstraete, Clin. chim. Acta *8*, 418 (1963).
[23] K. A. Grottum and N. O. Solum, Br. J. Haemat. *16*, 277 (1969).
[24] G. V. R. Born, J. Physiol., Lond. *209*, 487 (1970).
[25] R. D. MacKenzie, J. G. Henderson and J. M. Steinbach, Thromb. Diath. haemorrh. *25*, 30 (1971).
[26] I. Kobayashi and P. Didisheim, Thromb. Diath. haemorrh. *30*, 178 (1973).

Riassunto. Le piastrine di 3 diversi ceppi di ratti, contrariamente alle piastrine umane e di cavia, non sono aggregate (sia in vitro che in vivo) nè dalla ristocetina nè dal fattore von Willebrand bovino, porcino o umano, quest'ultimo reso aggregante previo trattamento enzimatico. Tale insensibilità, di cui si è potuta escludere l'origine plasmatica, potrebbe derivare dall'assenza, sulle piastrine di ratto, di un recettore per il fattore von Willebrand (acido sialico?); potrebbe essere così spiegata anche la mancata risposta delle piastrine alla ristocetina. Questi risultati suggeriscono che le piastrine di ratto possono essere un utile modello sperimentale per lo studio della sindrome di Bernard-Soulier nell'uomo.

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Low Virulence and Immunogenicity in Mice and in Rabbits of Variants of *Herpes simplex* Virus Resistant to 5-Iodo-2-Deoxyuridine

Previous research from this and other laboratories[1-3] demonstrated that the resistance to the inhibitory action of 5-iodo-2-deoxyuridine (IUdR) may be accompanied, in *Pox* viruses, by a marked decrease of virulence for receptive animals. Data here referred to show that this also holds for *Herpes simplex* viruses, and suggest a possible use of IUdR-resistant variants of *Herpes* virus in prophylaxis.

Material and methods. Human aneuploid HEp 2 cells (American type culture collection) and primary cultures of mouse embryo cells, which were grown in Eagle's MEM containing 7% calf serum, were used for in vitro experiments. Virus strains were *Herpes simplex* 1 (NIH) and one of its IUdR-resistant variants, obtained by serial passages in HEp 2 cells in the presence of increasing concentrations of IUdR.

In vitro experiments were carried out in cell monolayers (10^6 cells per sample) which were infected at 20 °C for 1 h with 5 infectious units (IU) per cell of either virus strain, washed 3 times in Eagle's MEM supplemented with 1% calf serum and incubated in the same medium at 37 °C. IUdR was added at time 0 after infection. 24 h later, cell cultures were frozen and thawed (−70 °C and +20 °C) 3 times and deprived of cell debris at 3,000 rpm for 5 min at 4 °C. The IU present in the supernatant liquid were titrated by the end point method (6 stationary tubes of HEp 2 cells per decimal dilution).

Part of the in vivo tests were carried out in young Swiss male mice, weighing 10 to 12 g, which were infected either i.p. (0.5 ml) or intracerebrally (0.02 ml). The onset of paralysis and lethality were registered.

Albino male rabbits weighing 2 to 2.5 kg were used in keratitis experiments. Eyes were anesthetized with 0.5% proparacaine, uniformly scratched 3 times with a hypodermic needle and infected with 2 drops of virus suspension or mock infected with Eagle's MEM. In rabbits infected twice, 2 or 30 days apart, the above technical procedure was repeated. The onset of conjunctivitis, keratitis, encephalitis and the lethality were registered. More details are given in the Tables.

Results. Data in Table I show that 10 serial passages in the presence of increasing concentrations of IUdR (from 0.1 up to 25 μg/ml) are sufficient to obtain from the original strain a variant which fully develops in the presence of IUdR concentrations 20 times higher than

Table II. Pathogenicity and immunogenicity of the IUdR-resistant variant of *Herpes simplex* virus in mice

1st injection (IU i.p.)[a]		2nd injection (IU i.c.)[b] (45 days apart)		Total No. mice paralyzed and dead / No. mice injected, at different times after the 2nd injection	
				10	30
—		HS[c]	10^3	18/18	18/18
—		HS	10^2	13/18	16/18
—		HS	10	3/18	4/18
—		HR[c]	10^3	6/18	8/18
—		HR	10^2	0/18	0/18
—		HR	10	0/18	0/18
HR[c]	10^6	HS[c]	10^3	0/18	0/18
	10^5	HS	10^3	0/18	0/18
	10^4	HS	10^3	0/18	0/18
	10^3	HS	10^3	3/18	3/18

[a] Tissue culture infectious units given i.p. [b] Tissue culture infectious units given intracerebrally. [c] See table 1.

Table I. Effect of IUdR on the growth of the IUdR-sensitive strain of *Herpes simplex* virus and one of its IUdR-resistant variants

Virus strain (5 IU per cell)[a]	Host cells	IU[a] produced in 24 h in the presence of IUdR (μg/ml)		
		0	2	20
Herpes simplex (HS)	HEp 2	3.3×10^7	$< 10^4$	$< 10^4$
IUdR-resistant HS variant (HR)[b]	HEp 2	1.6×10^7	5×10^7	3.3×10^7
Herpes simplex (HS)	Mouse embryo	1.6×10^6	$< 10^4$	$< 10^4$
IUdR-resistant HS variant (HR)[b]	Mouse embryo	8.3×10^5	1.6×10^6	3.3×10^6

[a] Infectious units, titrated in HEp 2 cells [b] Obtained by serially transplanting HS in HEp 2 cells, in the presence of IUdR (μg/ml): 0.1 – 0.2 – 0.4 – 0.8 – 1.6 – 3.2 – 6.4 – 12.8 – 25.6. The passages in the presence of 25.6 μg/ml were repeated 5 times and virus pool was then prepared by seeding the variant thus obtained in a IUdR – free Eagle's MEM.