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Species Variability in Platelet Aggregation Response to Different Agonists

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Summary

Conflicting data on platelet function in animal species are reported in the literature. In this study, the response of buffalo, horse, pig and sheep platelets to different agonists was assessed. Blood samples were collected from the jugular vein of six healthy subjects of each species and platelet-rich plasma was obtained by centrifugation. Platelet aggregation responses to increasing doses of adenosine 5′-diphosphate (ADP), arachidonic acid, collagen, platelet activating factor (PAF) and ristocetin were measured by a turbidimetric method. Horse platelets were the most responsive to ADP, collagen and PAF, whereas sheep platelets were the most responsive to ristocetin. The response to arachidonic acid varied least between species. PAF was the most effective agonist, inducing a maximum aggregation response at a concentration of 1 μM for platelets of each species. Conversely, concentrations of ristocetin higher than 1 mg/ml induced a maximum aggregation response only with sheep and horse platelets. The different responses of platelets from the four animal species to various agonists may reflect either (1) structural differences (including composition of the platelet membrane and presence of specific agonist receptors), or (2) activation of distinct signalling pathways by the agonist.

Introduction

Platelet aggregation represents the most important event during the formation of a platelet plug (Ruggeri, 1997). Two types of aggregation can be distinguished *in vitro*: primary aggregation that is reversible and occurs without the release reaction, and secondary aggregation that is irreversible and is associated with the release reaction (Born, 1962). Secondary aggregation is mainly mediated by endoperoxides (EPs) and thromboxane (Tx) A_2 formed from phospholipid-derived arachidonic acid, adenosine 5′-diphosphate (ADP) and Ca^{++} secreted from dense granules (Hamberg *et al.*, 1975; Gachet, 2000). The efficiency of these phenomena depends on the agonist and the concentration used to induce the aggregation response *in vitro*.

Common agonists differ in their requirement for extracellular cations and platelet cyclo-oxygenase activity for induction of secretion and irreversible platelet aggregation (Rao, 1994). Several pathways exist through which agonists cause platelet aggregation: in some cases, aggregation is a result of the effect of secreted ADP; in other cases, it may be considered a response to the activation of the arachidonate pathway that acts upon platelets; and, finally, through other mechanisms that are independent of secreted ADP or products of the arachidonate pathway (Rao, 1998).

In human medicine, platelets play major roles in the pathophysiology of disease processes such as atherosclerosis, thrombosis, metastasis, formation of malignant tumours, organ transplant rejection, inflammation and disseminated intravascular coagulation (Fuse, 1996). Alterations of platelet function also occur in many diseases of animals, for example, equine verminous arteritis, the systemic thromboembolism associated with endotoxaemia, and acute laminitis (Weiss *et al.*, 1995). Altered platelet function is documented in canine ehrlichiosis (Harrus *et al.*,

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1996), canine leishmaniosis (Valladares *et al.*, 1998) and bovine viral diarrhoea (Bezek *et al.*, 1994).

Studies of platelet function in animals are essential for understanding the process of haemostasis and thrombosis in human beings, and for developing new therapeutic approaches to a range of diseases. The use of human platelets in research may be limited by factors such as an inability to find subjects that are free of medications (e.g., aspirin) which can alter platelet function. New drugs and foreign implants that may contact blood (e.g., cardiovascular devices) may be tested in animals before their use in man. Laboratory animals are generally used for this purpose, but characterization of platelet function in animals other than these is limited. Quantitative studies of platelet functions in animals are also limited, and comparisons of data reported in the literature are difficult since different agonists and doses have been used (Addonizio *et al.*, 1978; Cargill *et al.*, 1983; Soloviev *et al.*, 1999).

The in-vitro aggregation of platelets in response to different stimuli is a useful tool for evaluating platelet activity in animal species, in health or disease. The aim of this study was to evaluate the specific activity of a range of agonists on the aggregation of platelets from four different animal species (buffalo, horse, pig, sheep). In particular, the effect of increasing concentrations of ADP, collagen, arachidonic acid, platelet activating factor (PAF) and ristocetin was assessed.

Materials and Methods

Animals

Buffaloes, sheep, pigs and horses (six animals for each species) bred on farms in the Campania area (Italy) were used. The animals selected for the study were females in seasonal anoestrus. All were healthy and had not received any medication for at least 1 month before the experiments.

Blood Collection

Blood was collected at 8.00 a.m. by jugular venipuncture with a 17-gauge needle. After discarding the initial 2 ml, 45 ml of blood were transferred to 50 ml centrifuge tubes containing 0.01 M trisodium citrate.

Chemicals

ADP sodium salt (A-2754), arachidonic acid sodium salt (A-8798), collagen type I from calf skin (885-1), PAF (P-4904) and ristocetin sulphate salt (R-7752), purchased from Sigma Chemical Company (St Louis, MO, USA), were of the highest quality available. Stock solutions of each agonist were prepared in phosphate buffer, pH 7·4, at the following concentrations: 1 mM ADP, 100 mM arachidonic acid, collagen 1 mg/ml, 10 μM PAF and ristocetin 20 mg/ml. These solutions were stored at −20°C, except for the collagen solution, which was stored at 4°C.

Platelet Aggregation

Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at different gravitational forces. Buffalo and sheep PRP were prepared by centrifugation at 250 ***g*** for 20 min, while equine and porcine PRP were prepared at 150 ***g*** for 15 min and 20 min, respectively, at room temperature (20–25°C). Autologous platelet-poor plasma (PPP) was prepared from PRP by centrifugation at 2000 ***g*** for 15 min. Platelets were counted in a haematocytometer chamber, with a phase-contrast microscope. The PRP counts were adjusted to a platelet count of 250 000/μl, by dilution with autologous PPP.

Aliquots of 225 μl PRP were incubated for 1 min at 37°C, before agonist (25 μl) at a given concentration was added. The aggregation profile was recorded for 5 min. All determinations were performed within 3 h of sampling. During this time, blood samples were kept at room temperature. PRP aggregation responses were measured with a Chronolog aggregometer (Haverton, PA, USA) coupled to a recorder. Quantification of the aggregation was determined by measuring the percentage difference in light transmission between PRP and autologous PPP reached in 5 min. Aggregation of 100% was considered to be equivalent to an 80–85% increase in light transmission. For each of the four species, the results reported represent the mean values of the percentage of aggregation ± SD of six animals, each of which was tested in triplicate. The reversibility of platelet aggregation induced by each dose of agonist was assessed from the shape of aggregation tracings, a monophasic curve indicating irreversible aggregation.

Results

ADP

Fig. 1 shows the percentage of platelet aggregation as a function of ADP concentration. ADP was a strong inducer of aggregation of platelets from all four species. However, platelets from the different species required different amounts of ADP to produce 100% aggregation. Horse and pig platelets were the most responsive to ADP, requiring concentrations of 2·5 μM and 5 μM, respectively, to reach the maximum percentage of aggregation. However, when

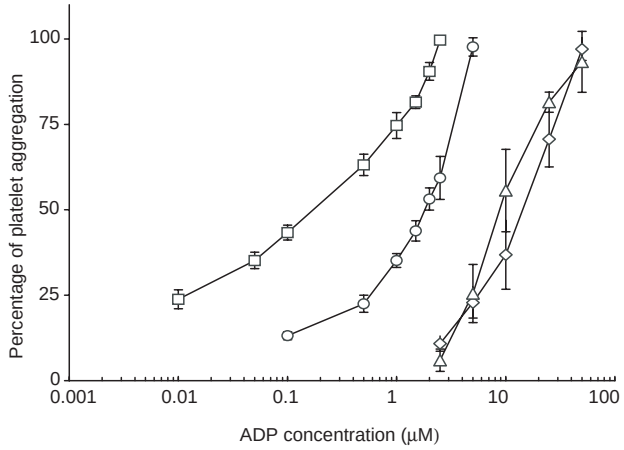

Fig. 1. Aggregation response, to increasing doses of ADP, of PRP from buffalo (△), horse (□), pig (◯) and sheep (◇). Error bars indicate SD. Each point represents the mean value for six animals, each of which was tested in triplicate.

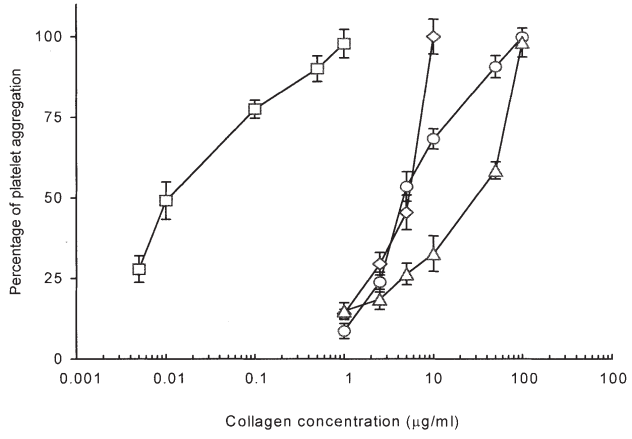

Fig. 2. Aggregation response, to increasing doses of collagen, of PRP from buffalo (△), horse (□), pig (◯) and sheep (◇). Error bars indicate SD. Each point represents the mean value for six animals, each of which was tested in triplicate.

concentrations of ADP lower than 2·5 μM were used, horse platelets showed a percentage of aggregation higher than that shown by pig platelets. Buffalo and sheep platelets required a concentration of ADP of 50 μM for the maximum percentage of aggregation. Furthermore, all concentrations of ADP tested caused irreversible aggregation of horse, pig and sheep platelets, whereas buffalo platelets showed a reversible aggregation at a dose lower than 10 μM (data not shown).

Collagen

Fig. 2 shows the percentage of platelet aggregation as a function of collagen concentration. Horse platelets

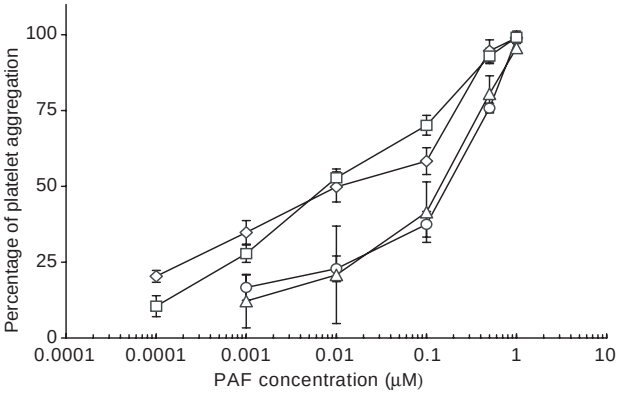

Fig. 3. Aggregation response, to increasing doses of PAF, of PRP from buffalo (△), horse (□), pig (◯) and sheep (◇). Error bars indicate SD. Each point represents the mean value for six animals, each of which was tested in triplicate.

were the most responsive to this agonist, the range of collagen doses used (0.005–1 μg/ml) being much lower than that required for the other species. Horse platelets reached 100% aggregation at a collagen concentration of 1 μg/ml, whereas platelets from sheep required a 10-fold higher concentration. Buffalo and pig platelets reached the maximum percentage of aggregation at a collagen concentration 100-fold higher than that needed for horse platelets. Furthermore, all doses of collagen tested caused irreversible aggregation of horse platelets, whereas collagen concentrations higher than 2·5 μg/ml were required to obtain irreversible aggregation of the platelets from the other animal species (data not shown).

PAF

Fig. 3 shows the percentage of platelet aggregation as a function of PAF concentration. This agonist showed strong aggregation of PRP from all species tested, inducing 100% aggregation at a concentration of 1 μM. However, two different trends were distinguished: horse and sheep platelets reached 50% aggregation at a PAF concentration of 0·01 μM, whereas buffalo and pig platelets required 0·2 μM to exhibit the same percentage of aggregation. All concentrations of PAF induced irreversible aggregation of platelets from each of the species tested (data not shown).

Arachidonic Acid

Fig. 4 shows the percentage of platelet aggregation as a function of arachidonic acid concentration. Arachidonic acid was less effective than ADP,

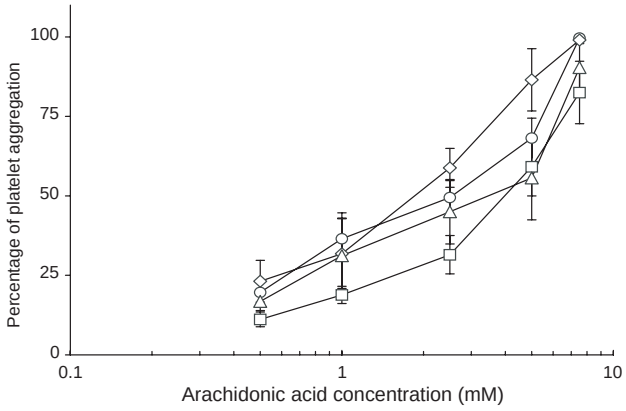

Fig. 4. Aggregation response, to increasing doses of arachidonic acid, of PRP from buffalo (△), horse (□), pig (◯) and sheep (◇). Error bars indicate SD. Each point represents the mean value for six animals, each of which was tested in triplicate.

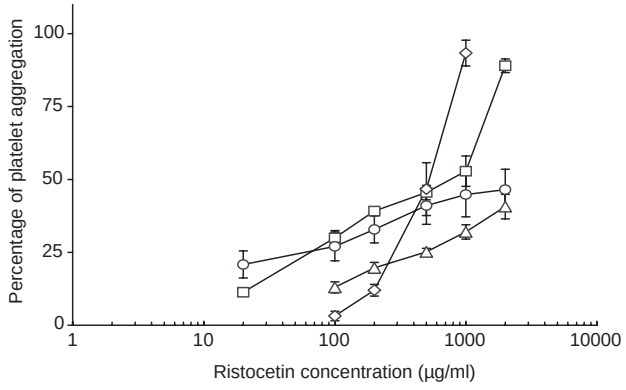

Fig. 5. Aggregation response, to increasing doses of ristocetin, of PRP from buffalo (△), horse (□), pig (◯) and sheep (◇). Error bars indicate SD. Each point represents the mean value for six animals, each of which was tested in triplicate.

collagen or PAF in inducing the aggregation of PRP from all four animal species. It gave the maximum response at a concentration of 7·5 mM. Even though the aggregation profiles were similar for all species, horse platelets were the least responsive.

Ristocetin

Fig. 5 shows the percentage of platelet aggregation as a function of ristocetin concentration. Ristocetin failed to induce a full response in all species tested. Sheep platelets gave 93% aggregation at a ristocetin concentration of 1·0 mg/ml, while horse platelets reached 89% aggregation at a concentration of 2·0 mg/ml. At this concentration, ristocetin induced 47 and 41% aggregation of pig and buffalo platelets, respectively.

Discussion

Platelet response to agonists in different animal species may be affected by differences both in the agonist mechanism of action and in platelet membrane structure. Structural differences in platelet membrane may result from several factors such as phospholipid content, arachidonic acid displacement as well as the expression of glycoproteins involved in signal transduction.

In this study marked differences in the aggregation responses to ADP, collagen, PAF, arachidonic acid and ristocetin of platelets from buffalo, horse, pig and sheep were highlighted. Of the platelets tested, those from the horse were the most responsive to all the agonists used, other than arachidonic acid. To reach the maximum percentage of aggregation, pig platelets needed twice as much ADP as that needed by horse platelets, and buffalo and sheep platelets 20 times as much. ADP, in all concentrations tested, induced irreversible aggregation of horse, pig and sheep platelets; in buffalo platelets, however, low concentrations of ADP caused reversible aggregation. This accords with a study by Marzec *et al.* (1975), who showed that when calf platelets were stimulated with ADP concentrations that normally cause irreversible aggregation in human platelets, only a primary aggregation response occurred; higher concentrations were needed to produce irreversible aggregation.

During ADP stimulation, substances such as ADP, serotonin and ATP may be released from the dense granules present in the cytoplasm of platelets. In addition to causing platelet aggregation, serotonin and ADP have been shown to interact with one another, or with other mediators, resulting in an enhancement of the response. Horse platelets contain large amounts of these substances (Meyers *et al.*, 1982). The platelets of the other animal species tested may require higher concentrations of external ADP for their aggregation because of (1) a lower content of ADP, (2) a different type of secretable nucleotide pool, or (3) different mechanisms for the release of the granule constituents.

In human platelets, the addition of ADP, in the presence of physiological concentrations of Ca^{++}, changes their shape, induces conformational changes of the membrane glycoprotein (GP) IIb/IIIa complex, and exposes the fibrinogen binding site on this complex. This process allows fibrinogen to bind to its receptor, which causes platelet aggregation (Hourani and Hall, 1994). Animal homologues of human GP IIb/IIIa have been detected on pig, dog, ruminant and horse

platelets by means of cross-reacting monoclonal or polyclonal antibodies (Perez de la Lastra *et al.*, 1997). Whether the different responses to ADP of the animal platelets tested are related to structural differences or to availability of GP IIb/IIIa complex needs to be investigated.

Horse platelets were the most active in responding to collagen stimulation, whereas buffalo platelets were the least responsive to this agonist. A previous report indicated that four times the concentration of collagen needed for human platelets was required to produce an equivalent calf platelet response (Marzec *et al.*, 1975). Other authors reported that collagen did not aggregate bovine platelets, but together with PAF it induced both aggregation and extensive TxB_2 formation (Kojima *et al.*, 1990). Sheep platelets synthesize prostaglandins (PGs) during collagen-induced but not ADP-induced aggregation (Leach and Thorburn, 1982); however, PG biosynthesis is much lower than in human platelets. In our experimental conditions, sheep platelets were more responsive than pig or buffalo platelets to collagen.

Collagen-induced platelet aggregation is dependent on endogenously generated TxA_2 (Kojima *et al.*, 1990). Degradation of several phospholipid classes by phospholipase A_2 in collagen-stimulated human platelets has been demonstrated (Takamura *et al.*, 1987). In addition to the role of phospholipase A_2 and C, however, the involvement of GTP-binding proteins, Na^+/H^+ exchange, lipocortin and actin filaments in collagen-stimulated activation of these enzymes has been established (Nakano *et al.*, 1989). The existence of two receptors for collagen on the platelet membrane has been suggested (Kamiguti *et al.*, 2000). Further studies are needed to establish whether the observed species differences in platelet response to collagen are due to (1) different availability of mediators of the signalling pathways activated by the interactions of collagen with its receptors, or (2) differences in structure or availability of collagen receptors on the platelet membrane in the four species.

Of the five agonists, PAF was the strongest in inducing the aggregation of platelets from all four animal species. However, at low concentrations of PAF, horse and sheep platelets showed a higher response than that shown by buffalo or pig platelets. Species differences in the response of platelets to PAF have been documented previously. Human, cow, horse, dog, cat, guinea-pig and rabbit platelets exhibit varying responses to PAF, whereas rat and mouse platelets are unresponsive (Namm *et al.*, 1982; Suquet and Leid, 1983; Liggitt *et al.*, 1984). Human PRP aggregates in response to PAF, but isolated human platelets do not do so without the addition of fibrinogen (Rao *et al.*, 1982). This has led to the categorization of PAF as a relatively "weak agonist" for human platelets, in that it induces release of platelet dense granules, but not of alpha granules. On the other hand, PAF induces aggregation and release in washed rabbit platelets and sheep platelets, even without the addition of fibrinogen (Moon *et al.*, 1990). Equine platelets are even more sensitive to PAF, and respond to this agonist independently of cyclo-oxygenase activity (Suquet and Leid, 1983).

Ligand binding studies indicate that specific PAF receptors, expressed on the surface of platelets, are responsible for the activity of this agonist (Valone *et al.*, 1982). Activation of these specific PAF receptors elicits diverse biochemical effects, including (1) activation of phospholipase C and A_2, which leads to hydrolysis of phosphoinositide and release of arachidonic acid, respectively, (2) increased cytosolic calcium concentration, and (3) activation of protein kinase C phosphorylation (Chao and Olson, 1993). In horse platelets prelabelled with (P^{32})phosphatidyl inositol, PAF initiated the rapid formation of labelled phosphatidic acid followed by an increase in phosphatidyl inositol (Lapetina, 1982). Studies on platelet PAF receptors expressed in some animal species have shown a quite different distribution and reorganization on the platelet surface (Chao and Olson, 1993). The differences observed in the aggregation response to PAF in the present study may therefore reflect structural and functional differences in PAF receptors expressed on platelet membranes of the animal species tested.

Platelet response to arachidonic acid showed low species-specificity; however, horse platelets were the least responsive to arachidonic acid stimulation. Arachidonic acid stimulates platelet aggregation after conversion to PGG_2 and PGH_2 and thence to TxA_2. The weak reversible aggregation response observed in horse platelets has been postulated to be a consequence of the amount of PG/Tx formed, or of the sensitivity of equine platelets to these substances (Meyers *et al.*, 1982; Clemmons *et al.*, 1985). Soloviev *et al.* (1999) reported that calf platelets did not respond to arachidonic acid: in the concentration used, it induced only a change in shape of the platelets, failing to induce platelet aggregation or ATP release. However, using a higher concentration of arachidonic acid, we observed aggregation of buffalo platelets.

Ristocetin was a weak agonist for all species tested. It failed to induce 100% aggregation of pig and buffalo platelets. However, sheep and horse platelets gave a significant aggregation response. Ristocetin has been reported to cause agglutination rather than a true aggregation of platelets. An agglutination reaction causes an increase in intracellular Ca^{++} concentration through two different mechanisms: in early

agglutination there is a Ca^{++} influx, and in late agglutination there is a discharge of Ca^{++} from internal stores (Bertolino *et al.*, 1995). In particular conditions, ristocetin mediates the interaction of the platelet membrane GPIb-IX-V complex with its adhesive ligand, von Willebrand factor, in the sub-endothelial matrix or plasma (De Luca *et al.*, 2000; Dong *et al.*, 2001). The exact mechanism by which ristocetin induces platelet aggregation in the animal species tested needs further investigation.

The differences in the responses of platelets from four animal species to different agonists highlight the distinct physiological behaviour of platelets from different species and provide the basis for further investigations on the mechanisms responsible for animal platelet activation.

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