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Production and characterization of a murine monoclonal antibody against human thromboxane synthetase

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Eicosanoids appear to have an important role in the actual momentary regulation of tissue blood flow. The function of constricting blood vessels by affecting the vascular tone has been assigned to thromboxane. Thromboxane synthetase, the enzyme responsible for the conversion of Prostaglandin-H_2 into thromboxane A_2, has been shown to be present in platelets, lung fibroblasts and the brain. Recently, thromboxane synthetase has been totally purified. The enzyme isolated from platelets appears to have a molecular weight of 58,800 Dalton and to belong to the group of cytochrome P450 proteins. In order to make a monoclonal antibody against thromboxane synthetase, BALB/c mice were injected four times i.m. with 10, 5, 5 and 4 µg of the platelet purified enzyme in complete Freund's adjuvant. The serum antibody titer against thromboxane synthetase in an ELISA was higher than 1:1000 after the second boost. One mouse received a fifth i.v. injection of 10 µg of the purified enzyme. One monoclonal antibody of the several hundreds of hybridomas screened in an ELISA revealed specific activity against thromboxane synthetase with a titer of 1:512 present in the culture supernatant. After recloning this reagent, called TÜ300, was used for the preparation of an immunoaffinity column, where it also reacted specifically. In immunoprecipitation experiments TÜ300 was able to precipitate a 58,000 D molecule. Also the biological activity of thromboxane synthetase could be blocked by monoclonal antibody TÜ300. In addition this reagent was employed in indirect immunofluorescence on leukemic cells employing a FACS IV cytofluorometer. Here specific staining of two megacaryocytic blast cell populations could be demonstrated. Thus TÜ300 appears to be a monoclonal antibody against human thromboxane synthetase.

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EFFECTS OF THROMBOXANE PATHWAY ANTAGONISTS ON HEPARIN-INDUCED PLATELET AGGREGATION (H-IPA) AND TESTING FOR HEPARIN-INDUCED THROMBOCYTOPENIA (HIT). L. D. Brace, *J. Fareed and D. Hoppensteadt, Univ. of Illinois at Chicago, Chicago, IL., USA and *Loyola Universtiy Medical Center, Maywood, IL USA.

We have reported that heparin and heparin fractions can induce platelet aggregation (PA) in a substantial number of normal healthy drug-free donors. H-IPA was shown to depend upon the molecular weight and concentration of the heparin preparation used, but its mechanism remains unknown. Therefore, we performed experiments with antagonists of the thromboxane pathway to determine whether arachidonic acid metabolites contribute to H-IPA. When indomethacin or 13-azaprostanoic (a thromboxane receptor anatagonist) was added to the PRP of donors whose platelets had been shown to aggregate in response to heparin, H-IPA was completely inhibited: heparin (bovine or porcine) caused 75% PA, while pretreatment with indomethacin or 13-APA reduced the response to 6%. Similarly, if the same donors ingested 650 mg aspirin 3 hours prior to phlebotomy, the PA response to heparin was reduced to approx. 10%. These results demonstrate that at least part of the mechanism of H-IPA is mediated through thromboxane generation. However, the mechanism by which heparin stimulates thromboxane production in platelets remains unknown.

In some patients, heparin is known to induce an immune response that causes severe thrombocytopenia (HIT) and is associated with arterial and venous thrombosis. Fratantoni, et al. (Blood 45:395-401, 1975) have introduced a PA method for the diagnosis of HIT. We have used a modification of this method to show that the PA observed when heparin is added to a mixture of normal donor PRP and HIT patient's serum or plasma can be inhibited by antagonists of the thromboxane pathway. When normal donor PRP was pretreated with indomethacin or 13-APA and then mixed with serum from a HIT patient (290 uL PRP:160 uL serum), the PA response to heparin was reduced from 75% to 10% or less. Similarly, if the PRP donors ingested 650 mg aspirin prior to phlebotomy, PA in the HIT test was reduced from 75% to 10% or less. Thus, the interaction of heparin with the antibody and platelets causes thromboxane generation and leads to PA. Cyclooxygenase specific antiplatelet drugs and inhibitors of thromboxane generation may be useful in the clinical management of HIT and H-IPA.

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ADRENALINE (ADR) INTERACTIONS WITH THROMBIN AND COLLAGEN - EFFECTS ON PLATELET ARACHIDONATE RELEASE AND 5HT SECRETION AND ROLE OF ENDOGENOUSLY FORMED THROMBOXANE A_2 (TxA_2). Y. Patel, S. Krishnamurthi and V.V. Kakkar. Thrombosis Research Unit, King's Coll. Sch. Med. Dent. Denmark Hill, London SE5 8RX, UK.

We have examined the effect of combinations of ADR + thrombin (T) and ADR + collagen (C) on platelet arachidonate release and 5HT secretion, and assessed the role of endogenously formed TxA_2 on these responses using indomethacin (I). Washed, human platelets prelabelled with [^{3}H]-arachidonic acid (AA) or [^{14}C]-5HT were used, ADR was added 10 sec before T or C and the reaction was terminated 3 min later. In the range 1-100µM, ADR induced no detectable aggregation or 5HT secretion but potentiated platelet aggregation when added with sub-threshold concentrations of T or C, which on their own induced no aggregation. At 2-4 fold higher concentrations of T and C (threshold for 5HT secretion), 5HT secretion and AA/TxB_2 release were also potentiated by ADR (1-10µM) by 30-50%. Pre-treatment of platelets with I (10µM) abolished threshold T and C-induced 5HT secretion, as well as its potentiation by ADR. However, approximately 2-fold and 5-fold higher concentrations of T and C respectively were able to induce 'I-insensitive'secretion, which was further potentiated by ADR. In I-treated platelets, C-induced AA release and its potentiation by ADR were also abolished suggesting a role for endogenously formed TxA_2. This was confirmed by addition of the TxA_2 mimetic, U46619 (0.3µM), which potentiated C-induced AA release in the presence and absence of ADR, even though it induced no AA release on its own or, in combination with ADR alone in the absence of collagen. The latter suggests agonist specificity regarding the ability of TxA_2 to synergistically stimulate AA release. Finally, unstirred platelets in PRP pre-incubated with ADR (10µM) for 120 min lost their responsiveness to ADR, when eventually stirred; however, these 'ADR-desensitised' platelets when washed and resuspended, were able to demonstrate synergistic effects on secretion when stimulated with ADR+T or ADR+C. This is analogous to the previously demonstrated ability of ADR to inhibit adenylate cyclase even in 'ADR-desensitised' platelets and re-inforces the separation regarding the mechanisms underlying the various effects of ADR on platelets.

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To investigate the hypothesis that abnormalities in prostanoid metabolism may be an aetiological factor in Raynaud's syndrome (RS) we have measured the stable metabolites 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2), using a radioimmunoassay. The table gives their levels (in ng/ml) and ratio (mean±SD) in healthy volunteers and in a group of patients with RS.

	Normals	Menstruating Females	Primary Raynaud's	Secondary Raynaud's	VWF
n =	25	5	13	10	8
6-keto-$PGF_{1\alpha}$	0.76±0.52	0.86±0.47	0.98±1.04	31.1±16.5	1.8±2.0
TXB_2	7.6±5.6	12.6±7.8	15.0±13.2	284±9.0	27.1±28.3
Ratio	10.3±3.1	14.0±3.5	16.6±3.6	10.4±3.8	15.6±4.7

In the control group there was no significant correlation between prostanoid levels and age, sex or smoking habit. In primary Raynaud's TXB_2 levels and the ratio tend to be elevated from our control values. Patients with Vibration White Finger Disease (VWF) have a similar eicosanoid profile to that of the primary Raynaud's group with elevated TXB_2 levels. One patient in this group had markedly raised 6-keto-$PGF_{1\alpha}$ and TXB_2 levels and later proved to have a circulating auto-antibody. Menstruating females in our study showed both elevated TXB_2 and ratios similar to the primary group. It is interesting to note that menstruating females have an increased sensitivity to cold with abnormal cold stress tests. Patients with Raynaud's syndrome secondary to a systemic disorder have markedly elevated 6-keto-$PGF_{1\alpha}$ and TXB_2 levels although the ratio of the two remains within our normal range. There is a significant difference between secondary Raynaud's and all other groups investigated in the study (Mann-Whitney U test $p = 0.04$). Further research into prostanoid metabolism may yield a greater understanding of the pathophysiology of Raynaud's syndrome. Therapy may best be aimed at altering the levels of these important local hormones.