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A monoclonal anti-thromboxane B_2 antibody

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A monoclonal antibody against thromboxane B_2 which may be used in standard fluid phase radioimmunoassays with a detection limit of around 40 pg and a binding affinity of 1.98×10^9 M^{-1} is described. Limited crossreactivity could be observed only with structurally closely related compounds such as 2,3-dinor-thromboxane B_2 (8.9%), thromboxane B_1 (15.7%) and thromboxane B_3 (39.7%). Detectable crossreactivity with 11-dehydro-thromboxane B_2, ω-carboxy-thromboxane B_2, ω-hydroxy-thromboxane B_2, prostaglandins of the D-, E- and F-type as well as metabolites of prostacyclin was lacking. The monoclonal anti-thromboxane B_2 antibody proved well suited for measuring the thromboxane B_2 content in tissue culture supernatants as well as in human serum.

Thromboxane B_2; Monoclonal antibody; Radioimmunoassay

1. INTRODUCTION

Thromboxanes (TXs) comprise a distinct subclass of eicosanoids known to derive from platelets and to regulate the function of these blood particles. The biologically active TXA_2 causes platelet aggregation and may play an important role in acute coronary disease [1]. Measurements of TXs have also attained clinical importance for monitoring graft rejection following kidney transplantation [2]. However, TXA_s is very unstable, with a half-life of 30–40 s at 37°C and physiological pH [3], and is quickly metabolized into the inactive but stable TXB_2. Therefore TXB_2 is widely used for indirect measurements of TXA_2 production in biological samples.

Many sensitive radioimmunoassays (RIAs) have been developed using polyclonal antibodies (PABs) from rabbit immunized with TXB_2. These PABs are very sensitive in this system and show little crossreaction with most prostaglandins, but, when tested, have been less specific against TXB_2 metabolites. Many PABs show considerable crossreaction with 2,3-dinor-TXB_2, which is present in blood [4,5], and, in even larger amounts, in urine [5,6].

Monoclonal antibodies (MABs) have been produced against prostaglandin E_2 [7–9], and can show good sensitivity and high specificity against related eicosanoids [7]. We, therefore, have developed a MAB against TXB_2 suitable for measurement of TXA_2 at biologically relevant concentrations in conventional fluid RIAs.

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2. MATERIALS AND METHODS

2.1. *Materials*

TXB_2 and other prostanoids were purchased from Sigma (Taufkirchen, FRG). Metabolites of TXB_2 were the generous gift of Dr C.O. Meese (Dr Margarete Fischer-Bosch, Institute of Clinical Pharmacology, Stuttgart, FRG), synthesized according to Fürst [10], Meese et al. [11], Schweer et al. [12], and Meese (in preparation). The radioantigen [5,6,8,9,11,12,14,15(n)-^{3}H]TXB_2 (spec. act. [15(n)-^{3}H]TXB_2 189 Ci/mmol) and the [^{3}H]TXB_2 assay system (TRK 780) were purchased from Amersham Buchler (Braunschweig, FRG).

2.2. *Hybridoma production and selection*

Approx. 8-week-old Balb/c mice were immunized each time with 20 μg TXB_2-BSA conjugate according to the method described [7]. Mice showing high titers of TXB_2 binding activity in serum were bled, the spleen removed and the spleen cells fused with the aminopterin sensitive myeloma cell line X63

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Table 1

Characteristics of monoclonal antibody against thromboxane B_2 (4E-TBR_1)

Prostaglandins	Crossreaction (IC_{50}) (%)
TXB_1	15.7
TXB_2	100 (0.40 ng)
TXB_3	39.7
2,3-Dinor TXB_2	8.6
11-Dehydro-TXB_1	0.5
11-Dehydro-TXB_2	0.7
ω-Carboxy-TXB_2	<0.1
ω-Hydroxy-TXB_2	<0.1
PGD_2	<0.1
$PGF_{2\alpha}$	<0.1
PGE_2	<0.1
6-Keto-$PGF_{1\alpha}$	<0.1
Sensitivity (ng)	0.04
Immunoglobulin subclass	IgG2a

Ag8.653 (G. Köhler, MPI, Freiburg, FRG) by the addition of polyethylene glycol 4000 (Merck, Darmstadt, FRG). After isolating hybridoma cells employing a selection medium containing hypoxanthine, aminopterin and thymidine, clones showing TXB_2 binding activity were selected. Subclones were produced on feeder layers of mouse peritoneal macrophages by two cycles of limiting dilution cloning. Cultures were expanded in Dulbecco's modified Eagle medium completed with sodium pyruvate, L-glutamine, 2-mercaptoethanol and 20% of fetal calf serum but without phenol red. The culture supernatants were collected and stored after lyophilization. For further use 1 mg of the lyophilized powder was dissolved in 1 ml of distilled water to prepare a stock solution of MAB.

2.3. *Characterization of MAB-TXB_2*

The MAB containing culture supernatants were checked for sensitivity and for binding specificity by standard fluid phase RIAs. The total sample volume of 1 ml contained about 10 μl MAB stock solution (~25% binding). Charcoal (100 μl of 3% solution) was used to separate the bound and unbound components. RIA with PAB (sensitivity 5 pg/sample) was carried out according to the kit description from Amersham with a sample volume of 300 μl and 1000 μl of dextran-coated charcoal used to separate bound from free TXB_2. The Ig subclass was identified using a hybridoma sub-isotyping kit (Calbiochem, Frankfurt, FRG). The MAB was also substituted for PABs in routine assays of the supernatants of macrophages stimulated to release eicosanoids (13) as well as in analysis of blood serum. For the latter experiments 2 ml venous blood was collected every 15 min using an Abbocath-T (20G × 32 mm, Abbott, Ireland), and directly dropped into tubes containing 100 μl indomethacin (10^{-4} mol/l). After 30 min incubation at 37°C and centrifugation the serum was frozen and stored at −70°C until the measurement of TXB_2 in fluid phase RIA. Platelet aggregation experiments were done with 1 ml platelet rich plasma stimulated by 5 μl arachidonic acid (76×10^{-3} mol/l) using a whole blood agrometer (Coulter, Krefeld, FRG). Aggregation was measured after addition of MAB-TXB_2 using 2–1000 μg of the lyophilized culture supernatant.

3. RESULTS

The characteristics of the new MAB-TXB_2 (4E-TBR_1) are compiled in table 1. The MAB belongs to the IgG2a subclass, and the affinity defined by

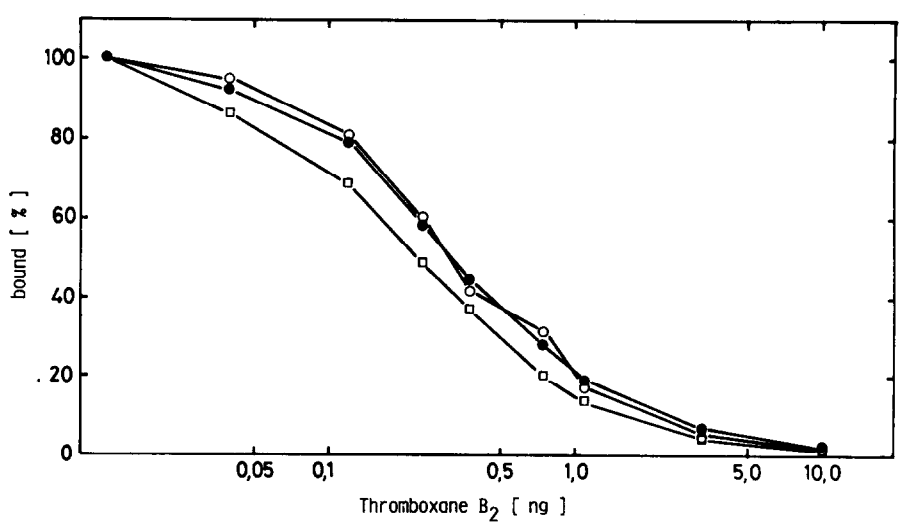

Fig.1. Standard curves describing the binding of TXB_2 to the MAB-TXB_2 (4E-TBR_1) using phosphate buffered saline alone (●), or supplemented with 10% plasma (○) and 10% serum (□) in a conventional fluid phase RIA.

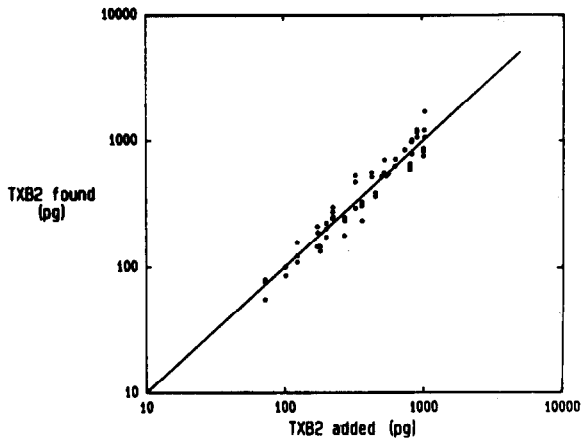

Fig.2. Recovery of TXB_2 with MAB-TXB_2 (4E-TBR_1) in a conventional fluid phase RIA.

Scatchard analysis is $1.98 \times 10^9\ M^{-1}$. Employing conventional fluid phase RIA with a 1 ml sample volume, the detection limit of TXB_2 was found to be about 40 pg.

We first examined the crossreactivity of the MAB against known metabolites of TXB_2. Modification of the α-chain usually causes high crossreactivity in PABs. Nevertheless, this MAB shows very low crossreactivity of 8.9% with the important TXB_2 metabolite 2,3-dinor-TXB_2. Reduction of the 5–6 bond to TXB_1 also results in low crossreactivity (15.7%). Upon analyzing metabolites with variations in the ring structure or with alterations in the ω-chain, we found less than 1% crossreactivity in each case. TXB_3 is an exception with a crossreactivity of 39.7%. Crossreactivity with prostaglandins was negligible.

To determine crossreactivity with the biologically active but unstable TXA_2, we tested whether our MAB-TXB_2 interferes with platelet aggregation initiated by arachidonic acid, since it is known that arachidonic acid-induced platelet aggregation is caused by TXA_2 [3]. Concentrations of MAB-TXB_2 which bound more than 55% TXB_2 within the first minute did not interfere with arachidonic acid-induced platelet aggregation in platelet rich plasma. This suggests that there is no prominent crossreactivity with TXA_2.

Some PABs change their sensitivity in assays containing biological fluids. In standard experiments the binding of TXB_2 to the MAB was not measurably influenced by the addition of up to 10% plasma or serum to the incubation mixture (fig.1). To validate the utility of the new MAB, we tested its accuracy in RIA by adding known amounts of TXB_2. Essentially 100% recovery was found over the tested concentration range (fig.2).

The calcium ionophore A23187 is widely used to investigate the effect of drugs on the production of prostaglandin E_2 and leukotrienes by mouse peritoneal macrophages. We have found this procedure to also produce TXs and have used it to compare our MAB-TXB_2 with a commercial PAB assay system for TXB_2. We examined two drugs, the non-steroidal anti-inflammatory drug indomethacin, an inhibitor of the cyclooxygenase enzyme, and dazmegrel, a selective inhibitor of the TX-synthetase enzyme (fig.3). Both inhibitors were studied at several concentrations. The data obtained from comparing the MAB and the PAB RIA system (see section 2) demonstrate a good correlation between the two RIA results. Both RIAs showed inhibition of the cyclooxygenase enzyme by indomethacin at a concentration of 10^{-6} M and the stepwise loss of inhibition up to a concentration of 10^{-9} M, with a correlation between the two systems of $r = 0.98$. The effect of dazmegrel as blocking agent was demonstrated up to 10^{-7} M, while there was no inhibition at 10^{-8} M. A close correlation of $r = 0.93$ was found for the results with MAB and PAB.

Finally, we examined the effect of ibuprofen, given at 500 mg p.o. to normal volunteers. TXB_2 was present in serum at a concentration of 100 ± 10 ng/ml. After drug administration TXB_2 declined to 1.0 ± 0.5 ng/ml at the time (60–120 min) of peak ibuprofen plasma levels ($n = 3$).

4. DISCUSSION

We have shown that it is possible to produce MABs against TXB_2 which are highly selective in comparison to PABs. TXB_2 metabolites as 2,3-dinor-TXB_2 are less than 10% crossreactive with our MAB. This is in contrast to results with PABs which show considerable crossreactivity with this metabolite (Anhut, personal communication [14], and anti-TXB_2 antibody TRK 780 Amersham, assayed in our laboratory). PABs against TXB_2 also show a relatively high degree (up to 13%) of crossreactivity with PGD_2 [14,15],

whereas the MAB-TXB_2 shows less than 0.1% crossreactivity. The ability to detect low amounts of TXB_2 in conventional fluid phase RIAs allows for the substitution of PABs by this MAB-TXB_2 in many assays. This can be advantageous due to the low crossreactivity particularly with respect to chemical substances with a similar ring structure or with a side chain similar to TXB_2. In addition, we have shown that our MAB may be used in routine RIA measurement procedures. Comparing the MAB with a commercial PAB in macrophage experiments we obtained good correlation (r = 0.96–0.98). The fact that both antibodies used in fluid phase RIA systems showed differences in the absolute quantities of TXB_2 measured in the tissue culture supernatants did not influence the determination of the inhibition range of the drugs, e.g. IC_{50}. Although differences were observed, they remain within the usual range of statistical error, possibly caused by the different characteristics of the MAB and the PAB and the different RIA methods. It would be of interest to determine the significance of the differences using independent analytical methods such as GC/MS. Finally, as we are in a position to produce this MAB in almost unlimited quantity and as its characteristics will not change from batch to batch as happens with PABs it may be of use in standardizing experimental results involving the measurement of TX metabolites.

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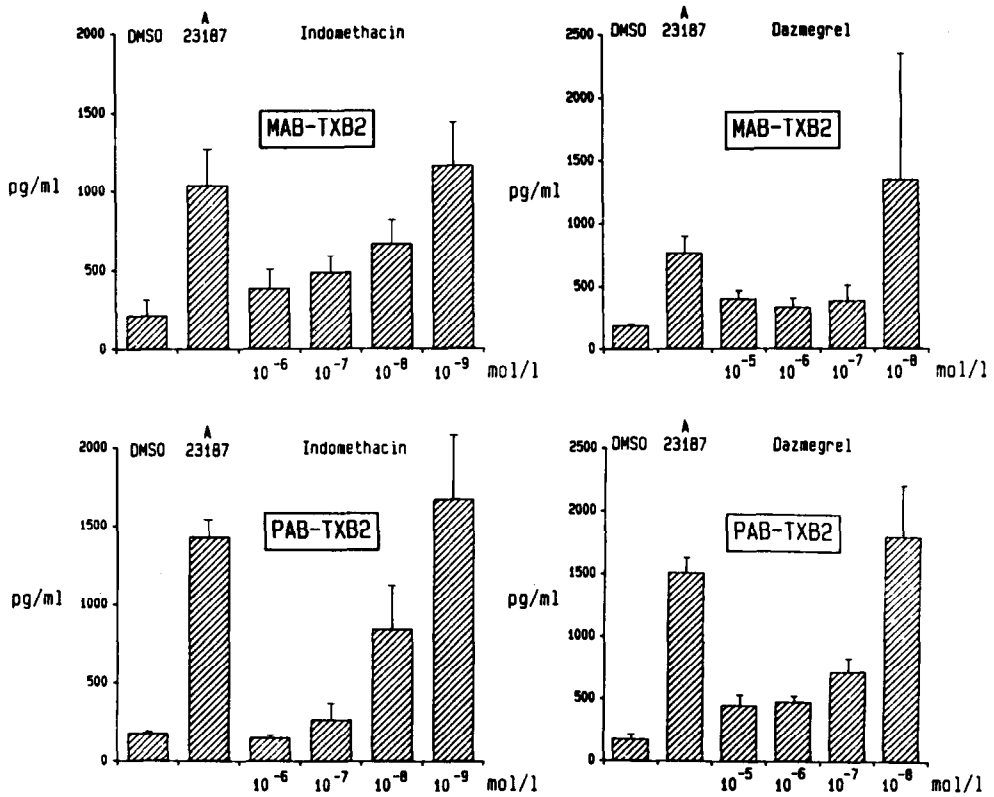

Fig.3. Effect of indomethacin and dazmegrel on A23187 (10^{-6} M)-stimulated release of TXA_2 (measured in terms of TXB_2-like activity) by mouse peritoneal macrophages using MAB-TXB_2 (4E-TBR_1) and polyclonal antiserum TRK 780 (Amersham) in different fluid phase RIA systems. One representative experiment with 5 culture dishes for each dose.

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