Production and Characterization of Polyclonal and Monoclonal Antibodies Against Human Thromboxane Synthase

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Polyclonal and monoclonal antibodies (MoAbs) were raised against human platelet thromboxane (Tx) synthase. Neither the antiserum nor the MoAbs inhibited the enzyme activity significantly. Three MoAbs, Tit 300, Kon 6, and Kon 7, were purified and further characterized. They are monospecific as shown by activity precipitation or Western blot analysis, and recognized different epitopes on Tx-synthase. Tit 300 could precipitate the enzyme and recognized conformational epitopes, whereas Kon 6 and Kon 7 only reacted in Western blots. Antibody Tit 300 can be used in immunohistology but shows no crossreactivity with Tx-synthase from other species. In human lung tissue staining with peroxidase, coupled Tit 300 was only found in alveolar macrophages.

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THROMBOXANE (Tx) synthase catalyzes the isomerization of prostaglandin endoperoxide (PGH2) to TxA2 and 12-hydroxyheptadecatrienoic acid (HHT). TxA2 is the major product of the cyclooxygenase pathway in platelets, and acts as a potent inducer of platelet aggregation and a strong vasoconstrictor. Its pathophysiologic roles are now the subject of active investigations in relation to thrombosis, vasospasm, and arteriosclerosis.

Tx-synthase activity has been measured in many tissues such as kidney, spleen, brain, and cell types such as platelets, macrophages, and lung fibroblasts. Its presence in other cell types remains unclear. Purification to homogeneity of the active enzyme from human platelets has been achieved by our laboratory. The protein was characterized as a cytochrome P450-enzyme with one heme per polypeptide of 58.8 Kd molecular weight. We also have studied the mechanism of thromboxane biosynthesis.

For a localization and quantitation in the various tissues, the immunochemical approach is ideally suited. Since the development of the hybridoma technology by Köhler and Milstein, the use of monoclonal antibodies (MoAbs) for characterization of proteins has found widespread application. In this report we describe for the first time the characterization of proteins has found widespread application. In this report we describe for the first time the development of the hybridoma technology by Köhler and Milstein, the use of monoclonal antibodies (MoAbs) for characterization of proteins has found widespread application. In this report we describe for the first time the generation and characterization of polyclonal and MoAbs directed against purified human platelet thromboxane synthase and their use in histochemical and Western blot analysis.

MATERIALS AND METHODS

Culture media and supplements were purchased from GIBCO Laboratories (Grand Island, NY), and fetal calf serum was from Biochroom KG (Berlin, FRG). Freund’s complete and incomplete adjuvants were obtained from Sigma Chemicals (St Louis, MO), polyethylene glycol 1500 from Boehringer Mannheim (Mannheim, FRG), and the tissue culture plates from Nunc (Roskilde, Denmark). All materials for column chromatography, including protein A, were supplied by Pharmacia (Uppsala, Sweden). 125-Iodine was purchased from Amersham Buchler (Brasuschweig, FRG). The iodination kit from Bio-Rad (Richmond, CA) and nitrocellulose paper from Millipore (Bedford, MA). Enzyme-labeled antibodies were obtained from Jackson Immunoresearch Lab (West Grove, PA), and bromo-chloroindolyl-phosphate and Nitroblue tetrazolium salt from Calbiochem (Frankfurt, FRG). Mayer’s hemalum solution was purchased by Merck (Darmstadt, FRG) and Aquamount for mounting by BDH Chemicals (Poole, UK). Animals were supplied by the Animal Research Institute, University of Konstanz, FRG. Myeloma line X63-Ag8.653 was kindly provided by Dr J. Kuhlmann and the antiserum to mouse IgG subclasses by Dr E. Weiler, Faculty of Biology, University of Konstanz. All solvents and other substances used were of analytical grade and obtained from Sigma.

Preparation of microsomes and Tx-synthase. Blood of different species was collected into 0.38% citrate solution and platelet-rich plasma was prepared by centrifugation at 120g for 3 minutes. Platelet microsomes and purified Tx-synthase were plated and mixed with peroxidase, coupled Tit 300 was only found in alveolar macrophages.

Preparation of a polyclonal antiserum to Tx-synthase. New Zealand rabbits were immunized by subcutaneous injection. The immunogen consisted of 0.5 mL solution of purified enzyme (50 µg) in 20% glycerol, 50 mmol/L K2HPO4, emulsified with an equal volume of complete Freund’s adjuvant. The rabbits were boosted four times with 25 µg of the antigen. Blood withdrawn from the marginal ear vein was allowed to stand overnight at 4°C and then centrifuged to 2,000g for 10 minutes. The IgG fraction in the serum was concentrated by ammonium sulphate precipitation, dialyzed against phosphate-buffered saline (PBS), and stored at −80°C.

Immunoprecipitation of Tx-synthase. Solubilized human platelet microsomes were prepared as described. Various dilutions of antibodies in 50 µL PBS were incubated with 40 µL solubilized microsomes (specific activity: 30 nmol/min/mg) for 1 hour on ice followed by a 30-minute incubation with 50 µL of 3% protein A Sepharose 4B solution. The tubes were then centrifuged at 10,000g for 2 minutes and the enzyme activity was measured in the supernatant.

Immunoprecipitation of 125I-labeled protein. Two hundred micrograms of solubilized human platelet microsomes were iodinated by the lactoperoxidase method with Enzymobeads (Bio-Rad, Richmond, CA). 125I-labeled protein was separated from free 125I by chromatography on a PD-10 column and then preadsorbed with protein A Sepharose and nonspecific immunoglobulin G (IgG). For immunoprecipitation, 125I-labeled microsomes (24 mCi) were added to 50 µL of MoAb and incubated for 1 hour at room temperature. Then 50 µL of a 30% protein A Sepharose solution was added and incubated for 30 minutes at room temperature. The tubes were centrifuged and washed three times with 1 mL washing buffer (50
mmol/L K$_2$HPO$_4$, 10% glycerol, 1 mol/L NaCl, 1% Lubrol PX). Finally, the pellets were resuspended in lysis buffer and subjected to gel electrophoresis on a 10% polyacrylamide gel as described by Laemmli. After fixing, staining, and destaining, the gel was dried and autoradiographed.

**Measurement of Tx-activity.** PGH$_2$ was prepared as described recently. Tx-synthase activity based on the ultraviolet light absorption of HHT at 234 nm was recorded continuously during incubation with PGH$_2$ in a cuvette as described.

**Preparation of anti-Tx synthase MoAbs.** Three BALB/c mice were injected peritoneally with purified enzyme (25 pg per injection) diluted in complete Freund's adjuvant, and boosted three times with purified Tx-synthase (10 pg per injection) diluted in incomplete Freund's adjuvant. Three days after the last intraperitoneal boost, spleen cells from the mouse with the highest serum titer of anti-Tx synthase antibody measured by enzyme-linked immunosorbent assay (ELISA) (see below) were harvested and fused with X63 Ag8.653 myeloma cells in a ratio of 1:2 in the presence of polyethylene glycol 1500. The cell suspension was plated into 96-well microtiter plates over mouse peritoneal feeder layers (10$^4$/well) in RPMI 1640 supplemented with 20% fetal calf serum and HAT medium. Screening of the plates for hybridoma cells secreting anti-Tx synthase antibodies was performed by ELISA. After the identification of positive clones, cells were subcloned two times by immunosorbent chromium release assay. Three clones were selected for use in this study.

**ELISA.** The reactivity of hybridoma culture supernatants was assessed by ELISA. Polyclonal antibodies to Tx-synthase in 50 mmol/L NaHCO$_3$ were adsorbed to wells (1 pg/well) in RPMI 1640 medium supplemented with 0.5 mol/L NaCl pH 8.3. Cyanogen bromide activation of Sepharose C-4B was performed according to the basic method outlined by Fuchs and Sela. After coupling rabbit anti-mouse IgG, column material was washed with 0.1 mol/L glycine pH 2.5 and equilibrated in 0.1 mol/L K$_2$HPO$_4$ pH 8.3. Hybridoma supernatant, 200 mL, was applied on a 10-mL bed volume, and bound antibodies were eluted by 0.1 mol/L glycine pH 2.5 and immediately neutralized with 1 mol/L Tris-HCl pH 8.0, dialyzed against PBS and stored at 80°C.

**Western blot.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% slab gels was performed as described by Towbin et al. To perform immunostaining, the nitrocellulose paper was soaked overnight in PBS containing 1% BSA and 0.02% NaN$_3$. After two washings with PBS, anti-Tx synthase antibody diluted in PBS with 0.1% BSA was incubated for 60 minutes at room temperature. Excess antibody was removed by washing three times in PBS containing 0.2% Tween 20. The paper was then incubated with peroxidase-labeled goat anti-rabbit IgG or alkaline phosphatase-labeled rabbit anti-mouse F(ab')$_2$ fragment, diluted in PBS/0.1% BSA for 60 minutes. After washing three times, the peroxidase reaction was initiated by incubating the nitrocellulose paper in 0.5 mg/mL 4-chloro-1-naphthol, 0.05% H$_2$O$_2$ in PBS pH 7.4. The alkaline phosphatase reaction was started by addition of bromochloro-indoly-phosphate (0.15 mg/mL) in 50 mmol/L Tris-HCl pH 9.5, 100 mmol/mL NaCl, 5 mmol/L MgCl$_2$. The reaction was terminated by washing out the substrate.

**MoAb isotyping and epitope analysis.** The isotype of the various anti-Tx synthase MoAbs was determined by ELISA. The different MoAbs were purified from cell culture by rabbit anti-mouse IgG immunoaffinity chromatography. The purified antibodies were diluted in 50 mmol/L NaHCO$_3$ for coating for 2 hours at room temperature in microtiter plates. After blocking two times with 1% BSA in PBS for 20 minutes, isotype-specific rabbit antisera were added for 1 hour. The washing and further processing were performed as described above for the ELISA screening assay. Epitope analysis was performed according to Figuet et al.

**Purification of MoAbs.** Large amounts of antibodies were produced by culturing hybridoma cells in roller bottles. RPMI 1640 medium was supplemented with 1 mmol/L pyruvate, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 pg streptomycin, 1% nonessential amino acids, 20% medium 199, and 5% fetal calf serum. For purification of the MoAbs, their Ig subclass was used. The IgG$_2a$, antibody was purified by protein A-Sepharose chromatography as described. The IgG$_1$ antibodies were purified by rabbit anti-mouse immunoaffinity chromatography.

The purification of anti-mouse IgG from rabbits was performed by precipitation with 40% ammonium sulphate saturation followed by ion exchange chromatography. Purified IgG (120 mg) was equilibrated in 0.1 mol/L NaHCO$_3$, 0.5 mol/L NaCl pH 8.3. Cyanogen bromide activation of Sepharose C-14B was performed according to the basic method outlined by Fuchs and Sela. After coupling rabbit anti-mouse IgG, column material was washed with 0.1 mol/L glycine pH 2.5 and equilibrated in 0.1 mol/L K$_2$HPO$_4$ pH 8.3. Hybridoma supernatant, 200 mL, was applied on a 10-mL bed volume, and bound antibodies were eluted by 0.1 mol/L glycine pH 2.5 and immediately neutralized with 1 mol/L Tris-HCl pH 8.0, dialyzed against PBS and stored at 80°C.

**Immunohistochemistry.** Tissue sections were washed with PBS and incubated in 10 mmol/L Na$_2$CO$_3$, 0.016% H$_2$O$_2$ in PBS for 10 minutes to inactivate endogenous peroxidases. After washing twice in PBS, preincubation was performed with 1% BSA in PBS, followed by incubation with polyclonal antibodies to Tx-synthase diluted in PBS/1% BSA. After washing three times, slices were incubated for 30 minutes at room temperature with goat anti-rabbit IgG peroxidase conjugate. After three additional washings, bound peroxidase was developed in the dark for 12 minutes with 3-amino-4-ethylcarbazole at pH 5.0 in acetate buffer containing 0.016% H$_2$O$_2$. Counterstaining with Mayer's hemalum solution and mounting in Aquamount mountant completed the procedure.

**RESULTS**

Polyclonal antibodies produced in rabbits in response to purified Tx-synthase bound, even at high dilutions, to the isolated Tx-synthase and reacted only with one protein in human platelet microsomes of the same molecular weight as determined by immunostaining (see Fig 2). The antibodies were able to precipitate the enzyme activity, but had no inhibitory effect on the activity even at high concentrations (data not shown).

The IgG fraction of the rabbit serum was concentrated by ammonium sulphate precipitation and used for coating in a sandwich ELISA (see Materials and Methods). The screening of hybridoma supernatants by this type of ELISA showed several stable monoclones. Three of them were used for further characterization. The specific binding of T6 300, Kon 6, and Kon 7 MoAbs to thromboxane synthase was demonstrated by an ELISA of these hybridoma supernatants (Fig
1). Serial dilutions of cell media exhibited a concentration-dependent binding of all three MoAbs to the enzyme. In controls, no binding of these MoAbs was detected in the absence of Tx-synthase. A control MoAb, directed against myosin light chain kinase exhibited no binding to Tx-synthase. To further characterize their monospecificity for Tx-synthase, the MoAbs were examined for binding to SDS-denatured Tx-synthase using the immunoblot technique. Human platelet microsomes and purified thromboxane synthase were electrophoresed by SDS-PAGE, transferred to nitrocellulose paper, and assayed for antibody staining with each of the three MoAbs and with the polyclonal antiserum (Fig 2). Kon 6 and Kon 7 as well as the rabbit antibody showed a single band corresponding to Tx-synthase. No other immunoreactive protein could be detected in human platelet microsomes. Tü 300 exhibited no binding to microsomal protein or Tx-synthase. Even in native as well as urea-PAGE with subsequent electrophoretic transfer to nitrocellulose paper, no binding to Tx-synthase could be achieved (data not shown). Together with the data in Fig 1, these results confirm the monospecificity of the MoAbs Kon 6 and Kon 7. The specificity of the MoAb Tü 300 to human Tx-synthase was established by immunoprecipitation.

Figure 3 demonstrates that Tü 300 is able to precipitate Tx-synthase activity in an immunoprecipitation assay with protein A Sepharose. The activity in the supernatant fraction was found to be gradually decreased at increasing concentrations of antibodies. A control antiserum that did not interact with Tx-synthase (see above) was without effect. If protein A Sepharose was omitted, no loss of activity was found in the supernatant, indicating that the MoAb did not react at the active site of the enzyme. To define the antigen precipitated by the Igs secreted by Tü 300, culture media from Tü 300 and from a control line were both incubated with a mixture of 125I-labeled proteins of solubilized human platelet microsomes (Fig 4). The resulting immunoprecipitates were solubilized with lysis buffer and then analyzed by SDS-PAGE and autoradiography. Only one radioiodinated protein was precipitated by Tü 300 that exhibited the same electrophoretic mobility as Tx-synthase. No iodinated pro-
teins were detected in the control immunoprecipitate. These results indicate that the Tü 300 hybridoma cell line secretes an Ig which, when bound to protein A Sepharose, will precipitate Tx-synthase activity and a single 58-Kd protein.

Ig subclass determination showed that Tü 300 belongs to the IgG₂ subclass, whereas Kon 6 and Kon 7 are of the IgG₁ type. No significant inhibition of Tx-synthase activity by coincubation of enzyme and antibody, even in high concentrations of MoAbs could be demonstrated. Epitope analysis showed that the MoAbs bind to different antigenic determinants on Tx-synthase. We suggest, based on the low additivity index of 38% for Kon 6 and Kon 7, that the epitopes of these antibodies partially overlap. The high index of 76% for Kon 6 and Tü 300 and 65% for Kon 7 and Tü 300 indicates that the antigenic determinant of Tü 300 is quite different from the others.

Larger amounts of MoAbs were produced in cell culture. Tü 300 was concentrated and purified by chromatography on protein A Sepharose. Kon 6 and Kon 7, which showed no reactivity with protein A, were purified by immunoaffinity chromatography with rabbit anti-mouse IgG coupled to Sepharose 4B. Figure 5 demonstrates the purity of the MoAbs isolated from cell culture supernatants. It also gives a hint for the diversity of the antibodies by their differences in the molecular weights of the light and heavy chains.

Fig 4. Immunoprecipitation of ¹²⁵I-labeled solubilized human platelet microsomes by MoAb Tü 300. Solubilized human platelet microsomes were radioiodinated by the lactoperoxidase method and incubated with MoAb Tü 300 and processed as in Fig 3. The resulting precipitates were washed and subjected to SDS-PAGE. Lane 1, control MoAb; lane 2, Tü 300 MoAb; lane 3, ¹²⁵I-labeled solubilized microsomes.

Fig 5. SDS-PAGE of purified MoAbs to human Tx-synthase. Electrophoresis was performed in a 10% slab gel. After electrophoresis, the gel was stained by Coomassie Blue R. The mobilities of molecular weight marker proteins are indicated. Lane 1, Tü 300 (5 µg); lane 2, Kon 6 (3 µg); lane 3, Kon 7 (3 µg).

To test for crossreactivity of Kon 7 with Tx-synthase from other species or with prostacyclin synthase, microsomal proteins were resolved by SDS-PAGE and analyzed by Western blot after transfer to nitrocellulose. Figure 6 gives evidence that purified Kon 7 was able to weakly detect swine and mouse Tx-synthase as compared with human Tx-synthase. The apparent molecular weight of the enzyme in these species appeared to be the same as for human enzyme. No crossreaction was found with rat, rabbit, or bovine platelet microsomes or with bovine aortic microsomes that represent a common source of prostacyclin synthase. Also, no reactivity was found with microsomes of human myometrium in which the occurrence of prostacyclin synthase has been reported. The purified MoAb Kon 6 exhibited the same pattern of reactivity (data not shown).

The MoAbs from Tü 300 seemed to be best suited for use in immunohistochemistry because it reacted with cut sections of frozen aggregated platelets (Fig 7A). MoAbs from Kon 6 and Kon 7 hybridoma cells expressed only very weak reactivity to platelets, prepared for immunohistochemistry. In human lung section Tx-synthase was localized by Tü 300 antibody in the alveolar macrophages by this method (Fig 7B). No other cell type in human lung exhibited antigenicity. With a cytospin preparation of human umbilical endothelial cells, no reaction with Tü 300 could be demonstrated.
DISCUSSION

The previously purified cytochrome P450-like enzyme Tx-synthase from human platelets was successfully used for the preparation of polyclonal and MoAbs. The antiserum obtained from the native enzyme in rabbits precipitated the enzyme in an apparently monospecific way but did not inhibit the activity. This antiserum was used for setting up an ELISA required for the screening procedure in the course of MoAb production.

By injecting several mice with the purified enzyme, we could isolate three hybridoma cell lines that secreted Iggs directed to Tx-synthase. We could demonstrate the monospecificity of these monoclonals by using different techniques. As in the rabbit antiserum, neither Tu 300, Kon 6, or Kon 7 inhibited the enzyme activity, suggesting that the active site is not affected and probably protected by a pocket form. The epitope for Tu 300 seems to be a conformational determinant, since it was destroyed during electrophoresis. In contrast, Kon 6 and Kon 7 gave the best staining in Western blots and obviously reacted with a polypeptide determinant exposed in the denatured form of the enzyme. From their low additivity index we conclude on an overlapping epitope reactivity. Probing Western blots of other species, only a weak crossreactivity with platelet microsomes from swine and mouse could be detected at a band of the same electrophoretic mobility as human Tx-synthase. This points to a large variability of Tx-synthase in its primary structure.

In view of the similarity of prostacyclin synthase with regard to its P450-nature and its substrate requirement compared with Tx-synthase, it was interesting to note no reactivity of Kon 7 with bovine aortic or human myometrium microsomes as a source of prostacyclin synthase. Further, Tu 300 was unable to crossreact immunohistochemically with human umbilical endothelial cells.

One of the major and most promising applications of the antibodies will be in immunohistochemistry. Preliminary experiments showed that human platelet aggregates gave an appropriate staining, and an application to human lung revealed the alveolar macrophages as the only cells with positive peroxidase staining. This finding agrees with earlier reports on the capacity of these cells to produce TxA. Also, Tx-synthase inhibitors can prevent the pulmonary vasoconstrictor response, the increase in pulmonary microvascular permeability induced by endotoxin, and also the leukotriene-induced bronchoconstriction in the guinea pig. In view of the important role of TxA in various pathophysiologic states, the further localization and quantitation of TxA-synthase will be a subject of future investigation.

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