An antibody present in normal human serum inhibits the binding of cytokines to their receptors in an *in vitro* system

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The presence of active transforming growth factor- β (TGF- β) in serum has not been widely accepted. In particular, although at least five studies have concluded that active TGF- β is present in normal human plasma and serum, assays that use the extracellular domain of the TGF- β type II receptor as a capture agent have given contradictory results. We show that there is an antagonist present in normal human serum which inhibits the binding of active TGF- β to the extracellular domain of the TGF- β type II receptor when it is coated on the well of an ELISA plate. This antagonist activity is due to a pool of immunoglobulins of the G₂, D and M classes. Moreover, we show that this same pool of immunoglobulins also recognizes the extracellular domain of the platelet-derived growth factor α -receptor, insulin-like growth factor-1 receptor and interleukin-3 re-

ceptor, by serial transfer of serum over the different receptors. In addition, the same immunoglobulin pool inhibits the binding of platelet-derived growth factor-AA to its receptor, in an analogous way to the inhibition of binding of TGF- β to its type II receptor. Circumstantial evidence suggests that the pool of immunoglobulins is recognizing a carbohydrate residue that is attached to the protein when it is synthesized by the mouse myeloma cell line, NSO, in which it is made. If the cytokine receptors are similarly glycosylated *in vivo*, then the presence of these antibodies in normal human serum may modulate physiological cytokine signalling.

Key words: carbohydrate, glycosylation, transforming growth factor- β , type II receptor.

INTRODUCTION

The transforming growth factor- β (TGF- β) superfamily consists of a number of related cytokines that have been implicated in many different physiological and pathological processes [1]. TGF- β is secreted as a latent complex with no known activity, consisting of TGF- β dimer linked non-covalently to its latency associated peptide [2]. It may also be found complexed with many other proteins, for example the latent TGF- β binding proteins (LTBPs) [3], α_2 -macroglobulin [4] and various extracellular matrix proteins [5,6].

The levels of TGF- β have been measured in many different tissues from many different species. TGF- β was found to be present in blood in 1983, when it was discovered that a large pool of biologically inactive TGF- β was present in platelets [7]. Since then, TGF- β has been identified in many different complexes in serum, platelets and plasma and measured using many different assays. The majority of these have been either ELISAs or bioassays. There are at least four commercially available ELISAs specific for TGF- β , and at least ten other ELISAs have been described in the literature to measure TGF- β [8–10]. Many different bioassays for TGF- β have also been described, using a variety of different cell lines and responses [11–13]. Despite (or perhaps because of) the use of these many different assays, a consensus has not yet been reached as to whether biologically active TGF- β (defined herein as TGF- β dimer which can bind the TGF- β receptor complex) is present in human plasma and serum.

We, and others, have previously published that active TGF- β is present in human plasma and serum [14–16]. However, the

R&D Systems Quantikine TGF- β 1 ELISA, that captures TGF- β using the extracellular domain of the TGF- β type II receptor (TGF- β sRII) produced in a mouse myeloma cell line, NSO, and uses a chicken polyclonal antibody as the detection reagent, does not detect any TGF- β 1 in human serum or plasma without prior activation. In order to investigate whether biologically active TGF- β was present in human serum or plasma, we have investigated the possibility that an inhibitor of the binding of TGF- β to its receptor is present in serum and plasma, and that this inhibitor explains why the Quantikine TGF- β 1 ELISA does not detect active TGF- β .

We show that a component of normal serum binds to the extracellular domain of the TGF- β type II receptor, and inhibits the subsequent binding of TGF- β . We show that this antagonist consists of a pool of immunoglobulins of the G₂, D and M subclasses.

MATERIALS AND METHODS

Materials

All of the cytokine soluble receptors were purchased carrier protein-free from R&D Systems Europe Ltd. (Abingdon, Oxon, U.K.). The anti-interleukin (IL)-4 antibody (3007.11) was purchased from R&D Systems and anti-platelet-derived-growth-factor (PDGF)-AA antibody (#06–130) was purchased from Upstate Biotechnology Ltd. (Lake Placid, New York, U.S.A.) The mouse monoclonal antibodies with high immunoglobulin subclass specificity were purchased from Skybio Ltd. (Wyboston,

Abbreviations used: IL, interleukin; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; PNS, pooled normal serum; sR, soluble receptor; TGF- β , transforming growth factor- β ; TGF- β sRII, the extracellular domain of the TGF- β type II receptor produced in NSO cells.

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Bedfordshire, U.K.) and used at a dilution of 1:10000 (approx. 100 ng/ml). The following clones were used: anti-IgG1, NL16; anti-IgG2, GOM1; anti-IgG3, HP6050; anti-IgG4, RJ4; anti-IgA, 2D7; anti-IgD, JA11; anti-IgE, 1109:1; anti-IgM, AF6. The purified human immunoglobulins were a gift from Skybio Ltd., except for human IgE, which was purchased from Biogenesis (Poole, Dorset, U.K.). The peroxidase-labelled anti-mouse IgG (A-0168) was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) and was used at a dilution of 1:10000 (approx. 380 ng/ml). The peroxidase-labelled anti-human IgG antibody (709-035-149) was purchased from Jackson ImmunoResearch (West Grove, PA, U.S.A.) and used at 1:20000 (22.5 ng/ml). Purified human IgG (009-000-003) was purchased from Jackson ImmunoResearch. K-blue substrate was purchased from Skybio Ltd.

TGF- β 1 Quantikine ELISA

The TGF- β 1 Quantikine ELISA was purchased from R&D Systems (DB100), and the assay performed according to the manufacturer's instructions, but with the following modifications. For the assay of antagonist activity, samples, that were not acid-activated, were incubated in the coated wells for 2 h. Following washing, as described in the manufacturer's instructions, wells were incubated with 2 ng/ml of TGF- β 1, reconstituted as described. Detection of bound TGF- β 1 was then performed as described in the manufacturer's instructions.

Ammonium sulphate precipitation of pooled normal serum

Pooled normal serum (PNS) was aliquoted and varying amounts of ammonium sulphate added as described previously [17]. Samples were then incubated overnight at 4 °C with rotation. Following centrifugation of the precipitated proteins, the supernatant was diluted 10-fold with PBS and concentrated 10-fold using an Ultrafree-4 centrifugal concentrator (Millipore U.K. Ltd, Watford, U.K.). This dilution and concentration was repeated twice more, such that the maximum concentration of ammonium sulphate in the final samples was 0.06 % of maximally saturated (approx. 2.5 mM).

Immunoglobulin binding assays and ELISAs

Wells were coated with 10 pmol of protein in 50 μ l of 100 mM Na₂CO₃, pH 8.5 for 2 h at 4 °C. Wells were washed three times without pausing with Tris-buffered saline (10 mM Tris/HCl, 154 mM NaCl, pH 7.4) containing 0.05 % Tween-20. Wells were then blocked with 350 μ l of PBS (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.2) containing 5 % (w/v) sucrose and 5 % (v/v) Tween-20 for 1 h on a plate shaker shaking at 500 rev./min. For the remaining steps see the appropriate descriptions in the Figure legends. Each of the incubations was 1 h, except for 'sample incubations', such as PNS or PNS fractions. Except where noted all incubations with normal serum were performed using pooled sera from 5 donors, two male and three female, aged between 22 and 31 years. Incubation volumes were 50 μ l for the samples and 200 μ l for each of the antibodies. Between incubations, each washing step consisted of three Tris-buffered saline/0.05 % Tween-20 washes as described above, except where the next incubation was serum, when a further wash with PBS was carried out prior to the incubation. Immediately following incubation with PNS, the number of washes was increased to five. Furthermore, immediately prior to colour development an additional wash with Tris-buffered saline was performed. Colour development was carried out with 200 μ l

of K-blue substrate for 2–20 min, and the reaction was stopped with 50 μ l of 2 M H₂SO₄. The absorbance of the wells were then read immediately at 450 nm using a Thermomax plate reader (Molecular Devices, Crawley, Sussex, U.K.).

RESULTS

The presence of an inhibitor of TGF- β binding to its type II receptor

If an inhibitor of the binding of TGF- β 1 to the extracellular domain of the TGF- β type II receptor (TGF- β sRII) coated on to the ELISA well were present in serum and plasma, it could prevent the interaction between ligand and receptor by binding to either the TGF- β 1 in the serum, or to the TGF- β sRII on the well. We investigated whether there was an inhibitor of the binding of TGF- β to its type II receptor present in serum that bound to the TGF- β sRII coated on a TGF- β 1 Quantikine ELISA plate. Wells were incubated firstly with PNS, and then, following washing, with TGF- β 1, to determine whether the binding of the TGF- β 1 to the plate was inhibited by a component present in serum that remained bound to the TGF- β sRII after washing. The results showed that the signal due to TGF- β 1 was reduced by 41.2 ± 1.1 % ($n = 5$; $P = 0.001$) by pre-incubating the well with PNS, but not reduced by incubating with high concentrations of protein (Figure 1). The TGF- β sRII antagonist activity could not be removed from the TGF- β sRII by a 2 h wash in either water or a 1 M salt solution (results not shown).

Purification of the inhibitory activity

For the initial purification of the TGF- β 1 sRII antagonist, ammonium sulphate precipitation of serum was performed. The resulting fractions were tested for inhibitory activity using the Quantikine assay, in the same way as described for untreated serum above. The inhibitory activity precipitated out of normal human serum at approx. 35–40 % of maximally saturated ammonium sulphate (Figure 2a). At this ammonium sulphate concentration, relatively few proteins precipitate out of serum,

Figure 1 Serum inhibits binding of TGF- β to its type II receptor

Sample was incubated in an R&D Systems Quantikine ELISA well. Serum was PNS, prepared as described in the Materials and methods section. HSA was human serum albumin, 45 mg/ml in PBS. Following washing three times with wash buffer, the wells were incubated with TGF- β 1 at 2 ng/ml. Following washing, the bound TGF- β 1 was detected using the detection antibody provided in the Quantikine assay. Values are means $\pm$ S.E.M. from five separate experiments. *The amount of TGF- β 1 binding to the capture reagent was significantly reduced by pre-incubation by PNS ($P = 0.001$, Student's unpaired *t*-test).

Figure 2 Serum inhibition of TGF-β1 binding to its receptor is due to an immunoglobulin

(a) Following precipitation of proteins from PNS by increasing concentrations of ammonium sulphate, supernatants were tested for their ability to block binding of TGF-β1 to its receptor, using the method described in the Materials and methods section and in the legend to Figure 1. (b) Assay of inhibitory activity of purified immunoglobulin compared with pooled serum. Values are mean ± S.E.M. from three separate experiments.

one class of which are immunoglobulins [17]. Therefore, to investigate whether the inhibitor of the TGF-β1 Quantikine ELISA was an immunoglobulin, we determined whether human IgG was binding to the TGF-β sRII. Serum was incubated on the wells of an ELISA plate pre-coated with TGF-β sRII and, following washing, bound human IgG was detected using an anti-human IgG peroxidase-conjugated antibody. Human IgG was found to bind to TGF-β sRII, and, furthermore, purified IgG at 8 or 16 mg/ml (approx. the concentration found in normal human serum [17]) inhibited the binding of TGF-β1 to the Quantikine assay, albeit to a lesser extent than serum (Figure 2b and results not shown).

Two different groups [18,19] have demonstrated previously the presence of complexes of TGF-β bound to IgG. It is therefore possible that the TGF-β sRII antagonist activity could be due to the presence of TGF-β-IgG complexes in the purified human IgG preparation binding to the TGF-β sRII through the TGF-β, but not being recognized by the Quantikine detection antibody because of the IgG. In order to investigate this possibility, we measured the concentration of TGF-β1 in our preparation of human IgG using three commercially available TGF-β1 ELISAs (R&D Systems, Genzyme and Amersham). The levels of TGF-

Figure 3 Subclasses of IgG that bind to the TGF-β sRII

Wells of an ELISA plate were coated with TGF-β sRII at 10 pmol/well. Wells were incubated with 50 µl of PNS, and bound immunoglobulin was detected using the mouse anti-immunoglobulin antibodies followed by an anti-mouse IgG peroxidase-conjugated antibody. Controls were performed omitting the primary antibody, the secondary antibody or the serum.

β1 in the preparation of purified human IgG varied depending on the assay used, from 0–138 pg/ml. Therefore there is a possibility that some TGF-β may be present in our IgG preparation and that this may be in the form of TGF-β-IgG complexes. However, based on the maximum detected concentration of TGF-β (138 pg/ml), the amount of TGF-β added to the wells with the IgG preparation would be 27 pg, or 1.1 fmol. This is likely to be substantially less than the amount of coated TGF-β sRII, since the binding capacity of ELISA wells is typically of the order of 1 µg. Furthermore, this is approx. 7% of the amount of TGF-β standard added in the second stage of the assay to detect unoccupied TGF-β sRII. Therefore, the presence of some TGF-β-IgG complex may account for some of the detection of IgG bound to the TGF-β sRII, but because of the relatively small amount of TGF-β in our human IgG preparation, the presence of TGF-β-IgG complexes cannot account for the 40% inhibition in the binding of active TGF-β to its receptor.

Characterization of the anti-TGF-β sRII

To determine which isotypes of human immunoglobulin bind to TGF-β sRII, we used a panel of mouse monoclonal detection antibodies highly specific for different human immunoglobulin isotypes (results not shown). The human immunoglobulin isotypes that bound to TGF-β sRII were of the G2, D and M classes (Figure 3).

The presence of more than one isotype of human immunoglobulin binding to the TGF-β sRII-coated well could be due to either of two possibilities. First, each of the immunoglobulin isotypes could be binding independently to the TGF-β sRII, or, secondly, IgG2, IgD and IgM could be binding to each other in the same way in which IgM rheumatoid factor binds to the Fc region of human IgG [20], with just one or two of the immunoglobulin isotypes binding directly to the TGF-β sRII. To test this, we coated the wells of ELISA plates with purified human immunoglobulins and incubated them with serum. The serum immunoglobulins that bound to the purified immunoglobulins coated on the ELISA well were then visualized using the mouse monoclonal class-specific detection antibodies. The

Figure 4 Binding of immunoglobulins from PNS to purified immunoglobulin subclasses

Purified immunoglobulins were coated on to the wells of an ELISA plate. Following the incubation of PNS, bound immunoglobulin was detected using the panel of mouse monoclonal detection antibodies and the anti-mouse IgG peroxidase-conjugated antibody. nd, not determined.

results (Figure 4) show that in normal serum there is noticeable binding of some of the serum immunoglobulins to purified immunoglobulins of different classes. Of those immunoglobulins that bind to wells coated with TGF- β sRII, a small amount of serum IgD binds to coated IgG2 and a small amount of IgM binds to purified IgD. However, this binding is unlikely to account for the large signals seen in Figure 3, since the amount of purified human immunoglobulin coated on the well in this experiment is substantially more than the total amount of IgG2, IgD or IgM binding to TGF- β sRII.

The binding of the serum immunoglobulins to TGF- β sRII is lost when the serum is diluted, with the signal from IgG2 and IgD reaching background at about a 1:8 dilution (Figure 5). However, this may be because the affinity of these immunoglobulins is low, or because the amounts of these immunoglobulins are low. To estimate which of these possibilities is likely to be correct, serum was incubated on wells coated with TGF- β sRII for 2 h and then the serum was removed. This serum was then incubated on a further set of wells, which had also been coated with TGF- β sRII. The results show that there was a marked reduction in the amount of IgG2 binding to the second set of wells, and a smaller reduction in the amount of IgD and IgM binding to the second set of wells (Figure 5b). This demonstrates that it is likely to be the amount of IgG2 that limits the signal when serum is diluted, and it can be calculated that the concentration of the IgG2 that binds TGF- β sRII in PNS is approx. 400 nM.

b

Figure 5 The concentration of anti-TGF- β sRII antibodies in PNS is low

(a) Dilution series of anti-TGF- β sRII in PNS. Since the dilution of anti-TGF- β sRII was shown to vary depending on the diluent used (results not shown), we prepared some PNS filtrate (collected when PNS was concentrated using ultrafiltration with a 5 kDa cut-off filter) that was essentially protein-free, with which to dilute the serum. (b) Reduction in the levels of anti-TGF- β sRII when incubating PNS on two wells sequentially. The first well was coated with either no protein or TGF- β sRII, and the second with TGF- β sRII. The graph illustrates the levels of anti-TGF- β sRII detected in the second of the two wells, as a fraction of that detected in the first set of wells.

Immunoglobulins that bind to other cytokine receptors

To determine whether there are serum immunoglobulins that bind to the extracellular domain of other cytokine receptors, we investigated the binding of serum immunoglobulin to several other cytokine receptors: IL-3 receptor [21], IL-4 receptor [22], IL-10 receptor [23], insulin-like growth factor (IGF)-1 receptor

Figure 6 Immunoglobulin subtypes binding to the extracellular domains of various cytokine receptors

Wells were coated with 10 pmol of the extracellular domain of various cytokine receptors. Following incubation with 50 μ l of PNS/well, bound immunoglobulin was detected using the panel of mouse monoclonal detection antibodies and anti-mouse IgG peroxidase-conjugated antibody. Values are means $\pm$ S.E.M. from duplicate wells.

[24] and PDGF receptor α [25]. The extracellular domain of each of these receptors was coated on to the surface of ELISA plates (10 pmol/well), the wells were incubated with serum and the isotypes of any immunoglobulins binding to the receptors were determined using the mouse monoclonal detection antibodies. We found that no human immunoglobulins bound to either the IL-4 soluble receptor (sR) or to the IL-10 sR. However, the immunoglobulin isotypes binding to each of the other receptors were the same as those that bound to the TGF- β sRII (Figure 6).

To test whether the antibodies to the other cytokine receptors also inhibited the binding of their respective cytokine ligand to the receptor, further ELISAs were developed in which cytokines were captured by the soluble receptor and then visualized using a detection antibody. We have developed such ELISAs both for PDGF-AA and for IL-4. Pre-incubation with serum was found to inhibit the binding of PDGF-AA to the PDGF sR α by approx. 70% (Figure 7a), in the same way that serum inhibits binding of TGF- β 1 to its receptor. However, for IL-4, where no natural human serum immunoglobulin to the soluble IL-4 receptor has been identified, serum did not inhibit the binding of IL-4 to the IL-4 receptor (Figure 7b).

Are these pools of immunoglobulins distinct?

To investigate whether the anti-cytokine receptor immunoglobulins were monoclonal, oligoclonal or polyclonal, wells were coated with TGF- β sRII and individual sera incubated in the wells. Specific mouse monoclonal antibodies for human kappa

and lambda light chains were then used to determine the relative usage of the kappa and lambda light chains. For five of the six people in our study, the immunoglobulins binding to TGF- β sRII were predominantly of one light chain or another (> 2-fold difference in values; Figure 8, top panel), whereas antibodies against the bacterial protein streptokinase [26] in the same serum samples showed markedly less bias towards one light chain or the other (Figure 8, bottom panel). This suggests that the antibody response to TGF- β sRII is oligoclonal, since each person tended to show a bias towards one light chain or the other, but did have anti-TGF- β sRII immunoglobulins containing both light chains, whereas the antibody response to streptokinase is polyclonal. Furthermore, the light chain bias of anti-TGF- β sRII for each person is very similar to the light chain bias for the same individuals of anti-PDGF sR α (compare Figure 8, top and middle panels).

Two lines of evidence now suggest that the same pool of immunoglobulins bind to each of the different receptors. Firstly, the binding of the different immunoglobulin isotypes to each of the cytokine receptors is very similar in that IgG2, IgD and IgM predominate in each case. Secondly, the ratio of binding of immunoglobulins containing kappa and lambda light chains for an individual is similar from one receptor to another. Since there is no sequence similarity between TGF- β sRII, PDGF sR α , IGF-1 sR and IL-3 sR these data suggest that the epitope(s) to which this pool of immunoglobulins bind may be either not a simple linear amino acid sequence, or the epitope is composed not of amino acids, but of carbohydrate residues on the surface of the

Figure 7 A component of serum inhibits the binding of some other cytokines to their receptors

ELISAs were developed that were based on the principle of the R&D Systems TGF- β 1 Quantikine ELISA. Soluble receptors were coated on to the wells of an ELISA plate at 10 pmol/well. Following pre-incubation of the wells with either PNS or PBS control, the purified cytokine was added to the wells. Bound cytokine was detected using a non-neutralizing antibody raised against the cytokine and an appropriate secondary antibody. **(a)** Wells were coated with PDGF sR α , and bound PDGF detected using the anti-PDGF-AA antibody and an anti-rabbit peroxidase-conjugated antibody. The results show that although serum contains a small amount of PDGF-AA, the pre-incubation of serum on the PDGF sR α markedly reduces the binding of purified recombinant PDGF-AA to the PDGF sR α . **(b)** Wells were coated with IL-4 sR, and bound IL-4 detected using the anti-IL-4 antibody and the anti-mouse IgG peroxidase-conjugated antibody. Unlike **(a)**, no IL-4 was detected in serum using this ELISA, and it can be seen that pre-incubation of the IL-4 sR with PNS does not reduce the subsequent detection of purified recombinant IL-4 in this assay.

proteins. To test these possibilities, we performed several experiments. Firstly, we analysed the epitope of the anti-TGF- β sRII using SPOTs (simple precise original test system) technology in which a series of overlapping 10 amino acid peptides across the length of the TGF- β sRII were generated on a nitrocellulose membrane. No binding of serum immunoglobulins to any peptide across the length of the molecule was found (results not shown).

Secondly, we have expressed the extracellular domain of the TGF- β type II receptor linked to glutathione S-transferase (R2X) in *E. coli* previously [16]. We cleaved the glutathione S-transferase from the TGF- β sRII using thrombin and further purified the TGF- β sRII. This TGF- β sRII expressed in *E. coli* differs from the TGF- β sRII expressed in NSO cells in three ways. Firstly, it does not contain any carbohydrate residues; secondly, there is a single Leu $\rightarrow$ Met mutation compared with the published sequence and thirdly, the TGF- β sRII expressed in *E. coli* cells contains the signal peptide [16,27]. The TGF- β sRII from *E. coli* cells was coated on to wells at the same concentration (measured by sandwich ELISA using two anti-TGF- β sRII antibodies) as the TGF- β sRII from NSO cells. No human immunoglobulins were found to bind to the *E. coli* TGF- β .

Thirdly, we performed an experiment to test whether the immunoglobulins that bind to each of the receptors come from the same or different pools. Serum was incubated in wells coated

Figure 8 Light chain predominance of anti-TGF- β sRII, anti-PDGF sR α and anti-streptokinase antibody responses

Wells were coated with antigen at 10 pmol/well, and following incubation with individual sera, the light chain predominance of the immunoglobulins binding to the ELISA wells was determined using antibodies that were specific for one light chain over the other. Light chain predominance of antibodies against TGF- β sRII, PDGF sR α and streptokinase are shown.

with one or the other of the soluble receptors, and then transferred on to wells coated with the same or a different soluble receptor (Figure 9a). Irrespective of which of the receptors the serum was transferred over, the signal on the second of the two receptors was markedly reduced (Figure 9b). However, when serum was transferred over TGF- β sRII expressed in *E. coli* (to which serum immunoglobulins do not bind; see above), no reduction in signal was observed. The only correlation between the receptor over which the serum was transferred and the signal on the second receptor is that the stronger the signal on the first receptor, the weaker the signal on the second (results not shown). This experiment demonstrates that the pools of immunoglobulins that bind to each of the TGF- β , PDGF and IGF-1 soluble receptors are the same.

DISCUSSION

The description of immunoglobulins that bind to the TGF- β sRII which is used as the coating reagent of the R&D Systems Quantikine ELISA demonstrates that this ELISA is not suitable for measuring the levels of active TGF- β in undiluted serum. However, the manufacturer does not recommend the use of this assay to measure TGF- β in undiluted serum, but rather in serum

Figure 9 The antibodies binding to each of the cytokine receptors come from a single pool of antibodies

PNS was incubated consecutively on two wells, each coated with either TGF- β sRII (from NSO cells or *E. coli*), PDGF sR α , IGF-1 sR or with no protein coated on the well. (a) Schematic representation of the experiment. (b) The signal on the second of the two wells on which the PNS was incubated is given. The results demonstrate that when serum is pre-incubated on any of the soluble receptors except for TGF- β sRII expressed in *E. coli* the signal on the second of the two wells is reduced, regardless of the soluble receptor coated on the second well. nd, not determined.

that has been treated with the addition of an equal volume amount of 2.5 M acetic acid and 10 M urea to activate any latent TGF- β , followed by neutralization and dilution. At these dilutions, the effect of the anti-carbohydrate antibodies is reduced, and furthermore we have shown that acid/urea and then neutralization of serum destroys the inhibitory activity. Therefore, although the ELISA is not suitable for measuring TGF- β in neat serum, it is, however, suitable for use as recommended by the manufacturer. The activation step that is required, however, does make this assay unsuitable for measuring levels of active TGF- β in biological fluids.

This study has shown that a pool of immunoglobulins in human serum bind to antigen(s) present on the extracellular domains of some, but not all, cytokine receptors. However, the TGF- β sRII, PDGF sR α , IGF-1 sR and IL-3 sR α are generated in a mouse myeloma cell line, NSO, whereas the IL-4 and IL-10 sR are purified from baculovirus-transfected insect Sf21 cells. Furthermore the immunoglobulin pool does not bind to TGF- β sRII when expressed in bacteria, even though this protein does bind its ligand [16] and is recognized by two separate anti-TGF- β sRII antibodies, suggesting that it is correctly folded. Therefore the antigen(s) recognized by the immunoglobulin pool

is likely to contain some post-translational modification of the receptors, such as glycosylation, since they are expressed by the mammalian NSO cells, but not by the insect Sf21 cells or bacteria. Literature searches revealed that Sf21 cells can glycosylate proteins with both O- and N-linked oligosaccharides and that the N-linked oligosaccharides may be of either the simple (high mannose) or complex (containing sialic acid) types [28], suggesting that the Sf21 cells can glycosylate proteins in many, if not all, of the same ways as mammalian cells. However, there have been reports that Sf21 cells quantitatively under-glycosylate expressed human proteins [29]. In addition, NSO cells have been shown to generate glycoproteins containing α -linked galactose residues and with a high N-glycolylneuraminic acid content, both of which may be immunogenic in man [30,31]. Preliminary experiments have found no evidence of binding of serum immunoglobulin to N-glycolylneuraminic acid, nor does a 100-fold excess concentration of N-glycolylneuraminic acid inhibit binding of serum antibodies to the TGF- β sRII (results not shown). However, we have shown that the *Bandeirea simplicifolia* lectin BS-I-B₄, which binds specifically to α -galactosyl residues, binds to those receptors to which the immunoglobulin pool binds, but not to the IL-4 or IL-10 soluble receptors. Therefore, although we have not demonstrated conclusively that the epitope to which the pool of serum immunoglobulins bind contains carbohydrate, nevertheless a candidate for future investigation is α -galactosyl, an epitope to which antibodies have previously been identified in normal human serum [32].

Anti-carbohydrate antibodies have a role in the immune response to encapsulated bacteria [33,34]. The majority of anti-carbohydrate antibodies are of the IgG2 subclass [35], and our observations are consistent with this. We were not aware of the identification of anti-carbohydrate antibodies of the IgD class prior to this study, but this may reflect the general lack of knowledge regarding IgD. Although there is similarity regarding the properties of each of the classes of the anti-receptor immunoglobulins, it is not yet known whether the G2, D and M immunoglobulins are each binding to the same epitope(s). Nor yet is it clear which of the antibodies are responsible for the blocking of the binding of TGF- β to its receptor or of PDGF-AA to the PDGF sR α . However, purified IgG does show some inhibitory activity (see Figure 2b), suggesting that the IgG2 is at least partly responsible for the blocking of binding of TGF- β to its receptor.

Our use of undiluted serum is unusual in that the majority of studies which have investigated the presence of natural antibodies in human serum dilute the serum, and use the degree to which the serum can be diluted (the titre) as a combined measure of the prevalence and affinity of the antibodies. However, we feel that the ability to dilute a serum 100-fold and still be able to detect an antibody has no relevance in the intact organism. Furthermore, we have found that the diluent in which the serum is diluted can have a large effect on the characteristics of the dilution curve for the antibody (results not shown). Although serum is not present in the intact organism, in that it contains the contents of degranulated platelets, the serum proteins that were present in whole blood are at the same concentration in serum as in whole blood. Any antibody present in undiluted serum and binding to its antigen on an ELISA well will presumably also bind its antigen *in vivo*. In the present case, the IgG2 antibody is at a concentration of approx. 400 nM, or 60 μ g/ml (approx. 2% of total serum IgG2).

Although the presence of IgM that binds to the Fc portion of IgG (rheumatoid factor) is well characterized, we are not aware of a full analysis in the literature of the binding of different immunoglobulin subtypes to each other. We show in Figure 4

that IgD binds significantly to both IgG1 and to IgG3, and slightly to IgG2. Furthermore, IgG4 binds to IgG2, IgG1 binds to IgG3 and, less surprisingly, serum IgM binds to purified IgD. Any biological relevance of this cross-binding of different Ig isotypes to each other in serum is not known.

Any function of the pool of anti-receptor antibodies identified in this study *in vivo* is unknown. *In vitro*, using the cytokine receptors that have been generated in the NSO cell line, the immunoglobulin pool found in normal serum clearly inhibits the binding of the cytokine to its receptor. However, it is not yet known if this occurs *in vivo*. This will depend upon whether the epitope(s) that is clearly present in the human protein when expressed in mouse NSO cells is also present in the native human protein expressed *in vivo*.

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