

Identification of molecules involved in the ‘early pregnancy factor’ phenomenon

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Summary. An isolated preparation from ovine placental extracts which was active in the rosette inhibition assay mimicking the activity of the so-called ‘early pregnancy factor’ (EPF) has been shown to contain a 12 kDa polypeptide which could be partially resolved from low-molecular-weight active moieties. *N*-terminal amino acid sequence analysis of the polypeptide indicated that it was ovine thioredoxin, an identification confirmed by isolation and complete sequence analysis of the corresponding cDNA. The cDNA for human thioredoxin was expressed in *Escherichia coli* and the recombinant protein isolated and purified. Pure recombinant thioredoxin alone did not induce the expression of increased rosette inhibition titres (RITs) when tested in the rosette inhibition assay; but, when tested in combination with cell stimuli such as platelet-activating factor (PAF) or serum, it allowed the expression of increased RITs where none was achieved in its absence. Thioredoxin acted in the assay to reverse a refractory state normally induced by these stimuli, allowing lipoxygenase-dependent moieties also induced by the stimuli to exert their effects, resulting in the expression of increased RITs. Antibodies to recombinant thioredoxin removed from pregnancy sera the capacity to induce increased RITs, i.e. to express EPF activity, thus establishing a role for thioredoxin or thioredoxin-like proteins and associated molecules in the mechanisms which allow pregnancy sera to induce increased RITs. Based on a consideration of these and other results, a new model for the study of the EPF phenomenon is presented and discussed.

Keywords: early pregnancy factor; thioredoxin; molecular definition; man; sheep; mouse

Introduction

For 16 years the ‘early pregnancy factor’ phenomenon has defied molecular definition. First described in 1974 by Morton *et al.*, this phenomenon is concerned with the detection *in vitro* of a lymphocyte-modifying activity in maternal serum. The activity is detected within hours of fertilization, being induced by the release of platelet-activating factor (PAF) from the fertilized ovum and is present for at least the first two-thirds of pregnancy, continued detection being dependent upon the presence of a viable embryo or fetus (Orozco *et al.*, 1986; Morton *et al.*, 1987). This most interesting activity with potential applications in early pregnancy testing, for monitoring fetal well-being and in the study of fertility control, is detected in the rosette inhibition assay. When lymphocytes are exposed to heterologous red blood cells in the presence of complement, a small subpopulation of the lymphocytes binds red blood cells to form rosettes. The ability of these lymphocytes to form rosettes can be inhibited in a dose-dependent manner by antilymphocyte serum (ALS), and so for a given ALS a rosette inhibition titre (RIT) may be defined. When

lymphocytes are incubated in pregnancy sera prior to testing in this assay, an increased rosette inhibition titre is observed (Morton *et al.*, 1976). The ability of pregnancy sera to cause this increase has generally been ascribed (Morton *et al.*, 1977, 1987) to the presence in these sera of a so-called 'early pregnancy factor' (EPF). Despite the common usage of this term, we emphasize here, as we have in the past (Clarke & Wilson, 1985), that EPF is defined by an activity in a complex biological assay *in vitro* and it is likely that the molecular basis for the activity is multifactorial.

Notwithstanding this, and additional data indicating that a complex set of components may be involved (Clarke *et al.*, 1980; Morton *et al.*, 1980), many attempts have been made (Wilson *et al.*, 1983; Cavanagh, 1984, 1985; Bose *et al.*, 1989; Mehta *et al.*, 1989) to isolate EPF on the assumption that activity expression is due to the presence of a simple pregnancy-specific protein. Preparations have been reported from a number of sources with the polypeptide components displaying a variety of physicochemical properties. Common features have been the paucity of material isolated, restricted characterization and the lack of any convincing data linking specific molecules in the preparations to the events by which pregnancy sera induce increased RITs.

Recently, we (Clarke *et al.*, 1987) described a protocol for the isolation of a fraction from ovine placental extracts which was active in the rosette inhibition assay. The major protein component of the preparation was a 12 kDa polypeptide which tended to oxidize to form 25 kDa disulphide-linked dimers. Moreover, antibodies produced against this preparation removed all EPF activity not only from placental extracts but also from pregnancy sera. Here, we report the identification of the 12 kDa polypeptide as thioredoxin. The coding sequence for human thioredoxin has been isolated and expressed in *Escherichia coli*, enabling production of both wild type and mutant forms of the protein free of contaminants of eukaryotic origin. This has allowed us to establish a role for thioredoxin in the mechanisms which give rise to so-called EPF activity expression. In so doing, this work provides not only the first molecular information on the EPF mechanism but also a new model for the study of this phenomenon.

Materials and Methods

Analysis of the ovine placental preparation. A 200 µg sample of the ovine placental preparation of Clarke *et al.* (1987) was subjected to chromatography on a calibrated Bio Gel P30 column (0.9 × 92 cm) in 1M acetic acid, collecting fractions of 0.75 ml. Protein elution was monitored using the method of Krystal *et al.* (1985). To facilitate analysis in the rosette inhibition assay, equal aliquots of fractions were pooled (see Fig. 1b), freeze-dried and resuspended in the original volume of phosphate-buffered saline (PBS). Samples of 1:10 serial dilutions prepared in PBS/bovine serum albumin (BSA) (0.1 mg/ml) were tested for their ability to cause an increase in RIT in the rosette inhibition assay. For analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), aliquants of selected fractions were freeze-dried and dissolved in SDS sample buffer lacking DL-dithiothreitol (DTT). SDS PAGE was performed on 15% polyacrylamide gels and the gel subjected to silver staining as described by Clarke *et al.* (1987).

Protein sequencing. Aliquants of the 25 kDa fraction from the Bio Gel P30 profile (see Fig. 1a) containing ~150 pmol of 25 kDa disulphide-linked dimer were subjected to N-terminal amino acid sequence analysis using an Applied Biosystems Gas Phase Sequencer at the Brisbane Protein and Nucleic Acid Research Centre. The unique sequence shown in Fig. 2 was obtained with initial yields of N-terminal amino acids of 280–300 pmol. Aliquants of the 12 kDa fraction (see Fig. 1a) were also subjected to analysis over 8 cycles.

Isolation of rat thioredoxin cDNA. Based on the amino acid sequence of the 12 kDa ovine placental protein, two oligonucleotide probes were designed:

(5'dGCGGCGTCCAGGGCCTCCTGGAAGGCGTACTTGGACTCGATCTGCTTCAC)

and

(5'dAAGAAGGGCTTGATCATCTTGCAGGGGCCGCACCAGGTGGCGGAGAAGTC)

They corresponded to the noncoding strand sequence for amino acids 1–17 and 25–41, respectively (Fig. 2) and were end-labelled with $\gamma^{32}\text{P}$ -ATP and used to screen a rat liver cDNA library made in lambda gt10. Hybridization was performed in 10% (w/v) dextran sulphate, 1M-NaCl, 1% (w/v) SDS, 50mM Tris-HCl, pH 7.5, 5 × Denhardt's and 500 µg denatured sonicated salmon sperm DNA/ml at 42°C overnight. The filters were then washed in 6 × standard sodium citrate (SSC), 0.1% SDS at 50°C. The 550 bp *Eco*RI insert from one positive phage was excised and subcloned

into M13mp18 and M13mp19 for sequence analysis. Both strands were sequenced according to the dideoxy-chain-termination method of Sanger *et al.* (1977).

Isolation of human and ovine thioredoxin cDNAs. The 550 bp rat thioredoxin cDNA was used as a probe to screen a human liver cDNA library and an ovine adrenal cortex library. Hybridization was performed as above, but with the addition of 40% formamide; washing was carried out in $2 \times \text{SSC}$, 0.1% SDS at 42°C. The inserts from positive phage were subcloned and sequenced as described above.

Expression of the human thioredoxin cDNA in *E. coli*. A 600 bp fragment containing the human thioredoxin cDNA insert was subcloned into M13mp19 and used for mutagenesis. A 30-base oligonucleotide (5'-ACTCCAGCAGCCAC-CATGGTGAAGCAGATC) was synthesized and used to create an *Nco*I site containing the ATG sequence. Mutant phage were detected by hybridization to the ^{32}P -labelled oligonucleotide after washing in 3.4M tetra methyl ammonium chloride (TMACl) (Wood *et al.*, 1985). After complete resequencing of the clone, the insert was excised and subcloned into the expression plasmid pET8.H3, under control of the T7 promoter. This construct was transformed into *E. coli* strain BL21[DE3], which contains the gene for T7 RNA polymerase under *lac* control (Studier & Moffatt, 1986). Synthesis of T7 RNA polymerase was induced by the addition of 0.4mM isopropyl- β -D-thiogalactopyranoside (IPTG), resulting in rapid transcription of the human thioredoxin coding sequences. Cells were collected 3 h after induction and human thioredoxin was isolated.

Isolation of human recombinant thioredoxin. For purification of human recombinant thioredoxin expressed in *E. coli*, 2 g of cells was resuspended in 20 volumes of 0.05M ammonium acetate, 1mM EDTA, 0.5mM DTT, 5mM α -aminobenzamide, pH 5.0, and lysed by passage through a French press. After centrifugation of the lysate (100 000 g for 90 min, Beckman 60 Ti rotor), the supernatant was applied to a column (2.5 \times 30 cm) of CM cellulose and eluted isocratically with 0.05M ammonium acetate, 1mM EDTA, 0.5mM DTT, pH 5.0. As shown by the SDS PAGE analysis (see Results), pure thioredoxin elutes as a distinct peak between fractions 60 and 85. These fractions were pooled, concentrated to 1–2 mg/ml, dialysed against 0.02M sodium phosphate buffer, pH 8.0, containing 1mM EDTA and 0.5mM DTT and stored as aliquots at -30°C .

Production of antisera to human recombinant thioredoxin and solid-phase adsorptions with antithioredoxin. Specific antibodies to recombinant thioredoxin were prepared by immunization of rabbits with highly purified recombinant human thioredoxin using standard protocols. The gammaglobulin fractions from an antithioredoxin antiserum and its pre-immune control were prepared and coupled to Affi Gel as described by Clarke *et al.* (1987). For adsorption of sera, 200 μl of each sample was added to 100 μl of packed Affi Gel (test or control) and incubated at room temperature for 4 h with frequent mixing then overnight at 4°C. The mixtures were then centrifuged (23 000 g for 2 min) and the supernatants (test and control supernatant) were collected and stored as aliquots at -30°C . After washing the Affi Gels 6 times with 0.02M sodium phosphate and 0.6M NaCl, pH 7.4, and twice with 0.9% NaCl, the specifically bound material was eluted by resuspension of the gels in 0.4 ml of 0.5M acetic acid for 5 min with frequent vortexing. After centrifugation the pH 2 eluates (test and control) were collected, freeze-dried and dissolved in 200 μl PBS/BSA and stored as aliquots at -30°C ; 5 μl of 10-fold serial dilutions was taken of each fraction for testing in the rosette inhibition assay.

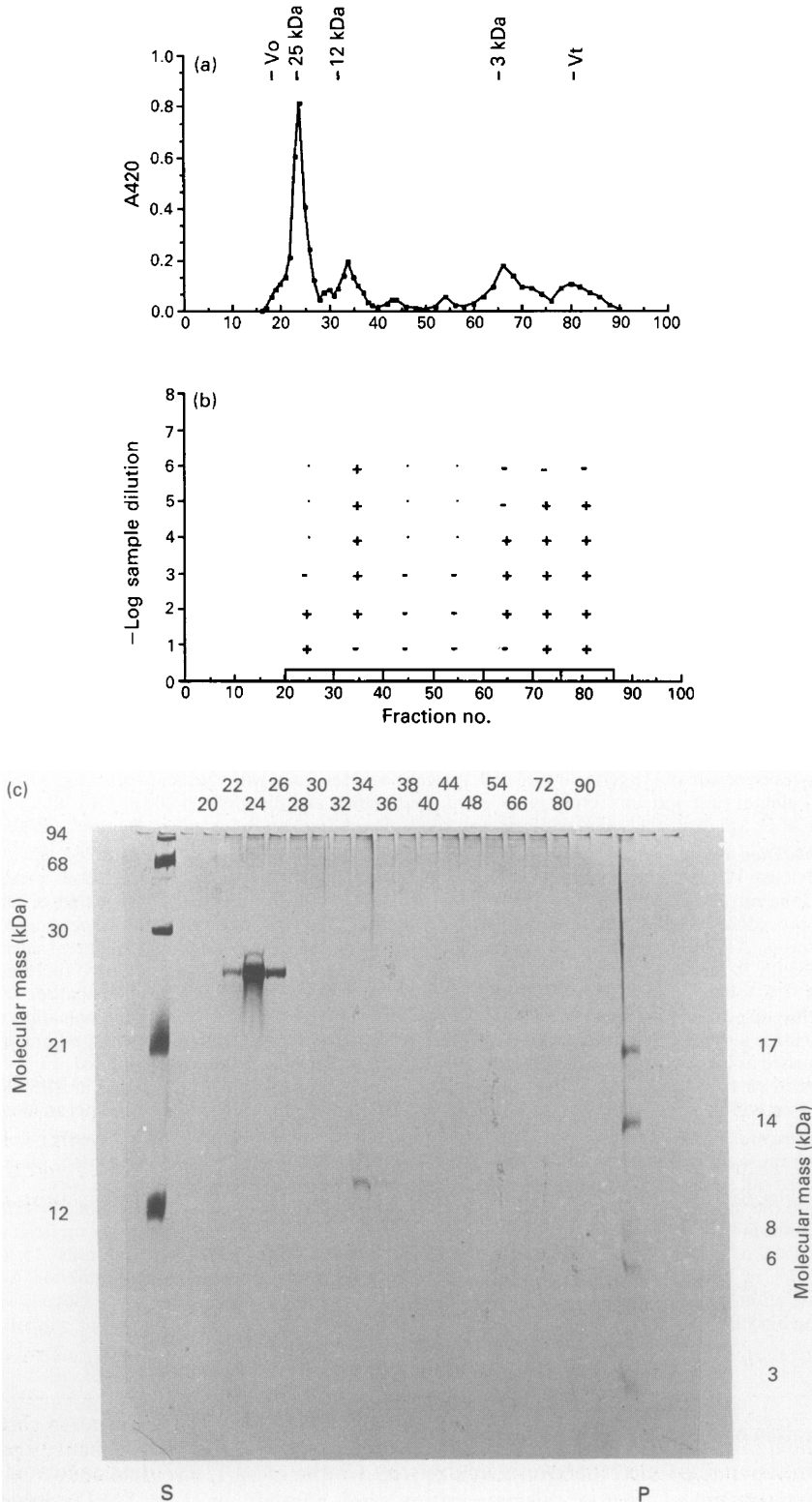
Rosette inhibition assay. The assays were performed using mouse spleen cells using the modified, efficient protocol described previously (Orozco *et al.*, 1986, 1990). To test substances in the assay, serial 1 in 10 dilutions were prepared in PBS/BSA (0.1 mg/ml) and aliquants (5 or 10 μl) added to 15×10^6 mouse spleen cells in a final volume of 200 μl PBS. In the standard procedure, the cells were then incubated for 30 min at 37°C before washing and dispensing into the anti-lymphocyte serum (ALS) dilutions. For the two-step protocol to test the order of addition of different substances, cells were incubated for 30 min at 37°C after addition of the first test substance, washed and subjected to a second 30-min incubation at 37°C following addition of the second test substance. At the end of the second incubation, the cells were washed and then dispensed into the ALS dilutions as usual. When thioredoxin was tested in combination with a fixed dose of male mouse serum or PAF, those doses were 0.5 μl and 5nM, respectively. In keeping with past practice, the RIT has been expressed as \log_2 [reciprocal dilution of ALS $\times 10^{-3}$]. So, if rosette formation is inhibited at 1 in 4×10^6 , the RIT is expressed as 12. This RIT of 12 was that obtained on control spleen cells incubated in PBS alone, so the observation of an RIT > 16 is considered to be significantly higher than the control and represents an increased RIT.

Ability of sera to stimulate the production of active S2 fractions. Mouse spleen cells (15×10^6) were incubated in the presence of serum samples (0.5 μl) in 200 μl PBS. After 30 min at 37°C the cells were sedimented, washed twice in Hank's balanced salt solution, resuspended in 200 μl PBS and incubated for a further 30 min at 37°C. The cells were sedimented and the supernatant, designated the S2 fraction, was collected; 10- μl aliquants of 1 in 100 serial dilutions of the S2 fractions in PBS/BSA were immediately taken for testing in the rosette inhibition assay on fresh spleen cells. To test the effects of indomethacin and nordihydroguaiaretic acid (NDGA), mouse spleen cells (15×10^6) were incubated at 37°C in 200 μl of PBS in the presence or absence of the desired concentration of inhibitor. After 15 min, 0.5 μl of mouse serum was added and the incubation continued for a further 30 min, after which the cells were washed and reincubated in PBS to generate the S2 fraction as described above.

Results

Characterization of the ovine placental preparation

Two-dimensional gel electrophoresis analysis by Clarke *et al.* (1987) had indicated that the 25 kDa disulphide-linked dimer of this preparation was a homodimer of the 12 kDa polypeptide, a



Ovine placental protein	V K Q I E S K Y A F Q E A L N S A G E K L V V V D F S A T W - G P - K M I K P
Thioredoxin Ovine	V K Q I E S K Y A F Q E A L N S A G E K L V V V D F S A T W C G P C K M I K P F F H S L S D K Y S N V V
Human	V K Q I E S K T A F Q E A L D A A G D K L V V V D F S A T W C G P C K M I K P F F H S L S D K Y S N V I
Rabbit	V K Q I E S K S A F Q E A L D S A G D K L V V V D F S A T W C G P C K M I K P F F H A L S D K F N N V V
Rat	V K L I E S K E A F Q E A L A A A G D K L V V V D F S A T W C G P C K M I K P F F H S L C D K Y S N V V
Chicken	V K S V G N L A D F E A E L K A A G E K L V V V D F S A T W C G P C K M I K P F F H S L C D K F G D V V
<i>Escherichia coli</i>	V K I I H L T D D S F D T D V L K A D G A I L V D F W A E W C G P C K M I A P I L D E I A D E Y Q G K L

F L E V D V D D C Q D V A A E C E V K C M P T F Q F F K K G Q K V S E F S G A N K E K L E A T I N E L I
F L E V D V D D C Q D V A S E C E V K C M P T F Q F F K K G Q K V G E F S G A N K E K L E A T I N E L V
F I D V D V A C K D I A A E C E V K C M P T F Q F F K K G Q K V G D F S G A N K E K L E A T I N D L L
F L E V D V D D C Q D V A A D C E V K C M P T F Q F Y K K G Q K V Q E F S G A N K E K L E A T I N E L V
F I E I D V D D A G D V A T H C D V K C M P T F Q F Y K N G K K V Q E F S G A N K E K L E E T I K S L V
T V A K L N I D Q D P G T A P K Y G I R G I P T L L L F K N G E V A A T K V G A L S K G Q L K E F L D A

Fig. 2. Comparison of the *N*-terminal amino acid sequence of the ovine placental polypeptide with the amino acid sequences of *Escherichia coli* and vertebrate thioredoxins. Our deduced amino acid sequence of human thioredoxin is shown, which differs from the published sequence of Wollman *et al.* (1988) at residues 38 (lysine instead of asparagine) and at 73 (methionine instead of threonine). The sequences of rabbit (Johnson *et al.*, 1988), rat (Tonissen *et al.*, 1989), chicken (Jones & Lund, 1988), and *E. coli* (Holmgren, 1968) thioredoxins are from the cited sources. * The active site dithiol.

conclusion now confirmed by separation and *N*-terminal amino acid sequence analysis of the molecules. The 25 kDa dimer and the 12 kDa monomer were resolved by gel-permeation chromatography (Fig. 1) and samples of each subjected to sequence analysis. The 25 kDa fraction yielded a unique sequence of 39 amino acids (Fig. 2) but with no assignments being made at positions 31 and 34. Sequencing of the 12 kDa fraction over eight cycles yielded a single sequence identical to the 25 kDa molecule. Comparison of the 39-amino-acid sequence with sequences in the data banks revealed a significant homology with bacterial thioredoxin (Fig. 2) particularly around the dithiol active site (* in Fig. 2) indicating that residues 31 and 34 of the ovine placental polypeptide were cysteines. These had not been detected initially as the samples of placental protein were analysed without prior carboxymethylation. More recently, the sequences of three vertebrate thioredoxins (chicken, rabbit and human) have been published (see legend Fig. 2) and we have also determined that of human and of two others, rat and ovine, allowing unequivocal identification of the ovine placental 12 kDa protein as ovine thioredoxin (Fig. 2). While rosette inhibition assays of the ovine placental preparation after gel-permeation fractionation indicated some activity expression coincident with the pools containing the 12 kDa polypeptide and its 25 kDa disulphide-linked dimer (Fig. 1b), there was also strong activity expression associated with fractions eluting near the total volume of the column (Fig. 1b). Nothing was visible from these fractions after silver staining of SDS PAGE gels (Fig. 1c), although iodination of these fractions resulted in ^{125}I incorporation into molecules of apparently low molecular weight as indicated by their inclusion in Bio Gel P2 columns and by their rapid migration on SDS PAGE gels. These observations suggested that the thioredoxin polypeptide was not the only component of the isolated fraction and so may not be solely responsible for the activity expression in the preparation. To help resolve these issues, human thioredoxin cDNA sequences were isolated and the coding sequences expressed in *E. coli*. The purified protein was tested in the rosette inhibition assay and the thioredoxin from recombinant sources was used to raise specific antibodies.

Production of recombinant thioredoxin

Based on the amino acid sequence of the 12 kDa ovine placental protein, two oligonucleotide probes were designed and used to isolate a 550 bp cDNA encoding rat thioredoxin (Tonissen *et al.*,

Fig. 1. Fractionation and analysis of ovine placental preparation by gel permeation chromatography on Bio Gel P30 (a) protein elution profile (b) rosette inhibition titre activity profile and (c) silver-stained SDS PAGE analysis of selected fractions. V_0 = void volume. V_t = total volume of column.

1989) from a rat liver cDNA library. This insert was then used as a probe for screening a human liver cDNA library to isolate a human thioredoxin clone. The deduced amino acid sequence of one such human clone differed in two places (see Fig. 2 and legend) from the previously published sequence of Wollman *et al.* (1988). Recombinant human thioredoxin was expressed in *E. coli* BL21 using a T7 RNA polymerase expression system. Human thioredoxin was produced as a soluble protein accounting for 15% of the total protein in induced cells and was purified to homogeneity using ion-exchange chromatography (Fig. 3a, b).

Role of thioredoxin in the rosette inhibition assay

Pure human recombinant thioredoxin was without activity in the rosette inhibition assay. When mouse spleen cells were incubated with this protein over a wide range of concentrations, the RITs determined were equivalent to those obtained after control incubations in PBS or nonpregnancy sera (Fig. 4a). However, we found that, while thioredoxin and nonpregnancy sera individually do not cause the expression of increased rosette inhibition titres, the combination does. When thioredoxin and male mouse serum were simultaneously added to mouse spleen cells, increased RITs were observed, dose-response data (Fig. 4a, b) showing that thioredoxin was extremely effective in co-operating with nonpregnancy serum components to induce activity expression. When mouse spleen cells were first incubated with male mouse serum alone, washed, then incubated with pure thioredoxin before testing, increased RITs were still observed over a comparable range of thioredoxin concentration (Fig. 4a) as when both were added simultaneously; but when, the two-step incubation was performed in reverse order, there was no change (Fig. 4a) in RITs above negative control values. This suggests that thioredoxin plays a permissive role subsequent to the action of serum components on the spleen cells and in this way the combination mimics the action of pregnancy serum ascribed to the so-called 'early pregnancy factor'.

The co-operation between thioredoxin and male mouse serum resembles our findings (Orozco *et al.*, 1990) that a combination of oestrus-specific mouse serum components and PAF could also increase RITs. Neither alone was effective, the combination was required. Again the effect was observed in a two-step incubation protocol only when a prescribed order of addition was followed, in this case, PAF being applied in the first step. Extensive studies (Clarke *et al.*, 1990a; Orozco *et al.*, 1990) revealed that PAF acts on the spleen cell population to stimulate the production of lipoxygenase-dependent soluble factors (termed S2 factors) which are capable by themselves of increasing RITs when applied to fresh (nonPAF stimulated) spleen cells. While PAF-stimulated spleen cell populations produce S2 factors, they are rendered refractory to the action of these compounds and so do not display increased RITs unless oestrous mouse serum components are also applied (see Orozco *et al.*, 1990). The oestrus-dependent components in this serum interact with the PAF-stimulated cells to reverse the refractory state, allowing the S2 factors to exert their effects and resulting in the expression of increased RITs. The similarities between the PAF/oestrous mouse serum combination and the serum/thioredoxin combination in inducing increased RITs, suggested that in the latter system the serum may be acting on cells in a similar fashion to PAF, i.e. inducing S2 factors and a refractory state, while the thioredoxin may be functionally equivalent to the components generated on interaction of oestrous mouse sera with PAF-stimulated cells, which act to reverse the refractory state. If so, it would be expected that mouse sera could induce the production of S2 factors and that thioredoxin could co-operate with PAF to induce the expression of increased RITs. This is indeed the case, as shown by the experiments described below.

Induction of an active S2 fraction by mice sera

To test the possibility that mice sera could induce the production of an active S2 fraction, mouse spleen cells were incubated with mouse serum samples for 30 min, washed and then subjected to a second incubation in PBS for a further 30 min. After the removal of the cells by centrifugation, aliquants of 100-fold serial dilutions of the supernatant (S2) were taken for testing on fresh

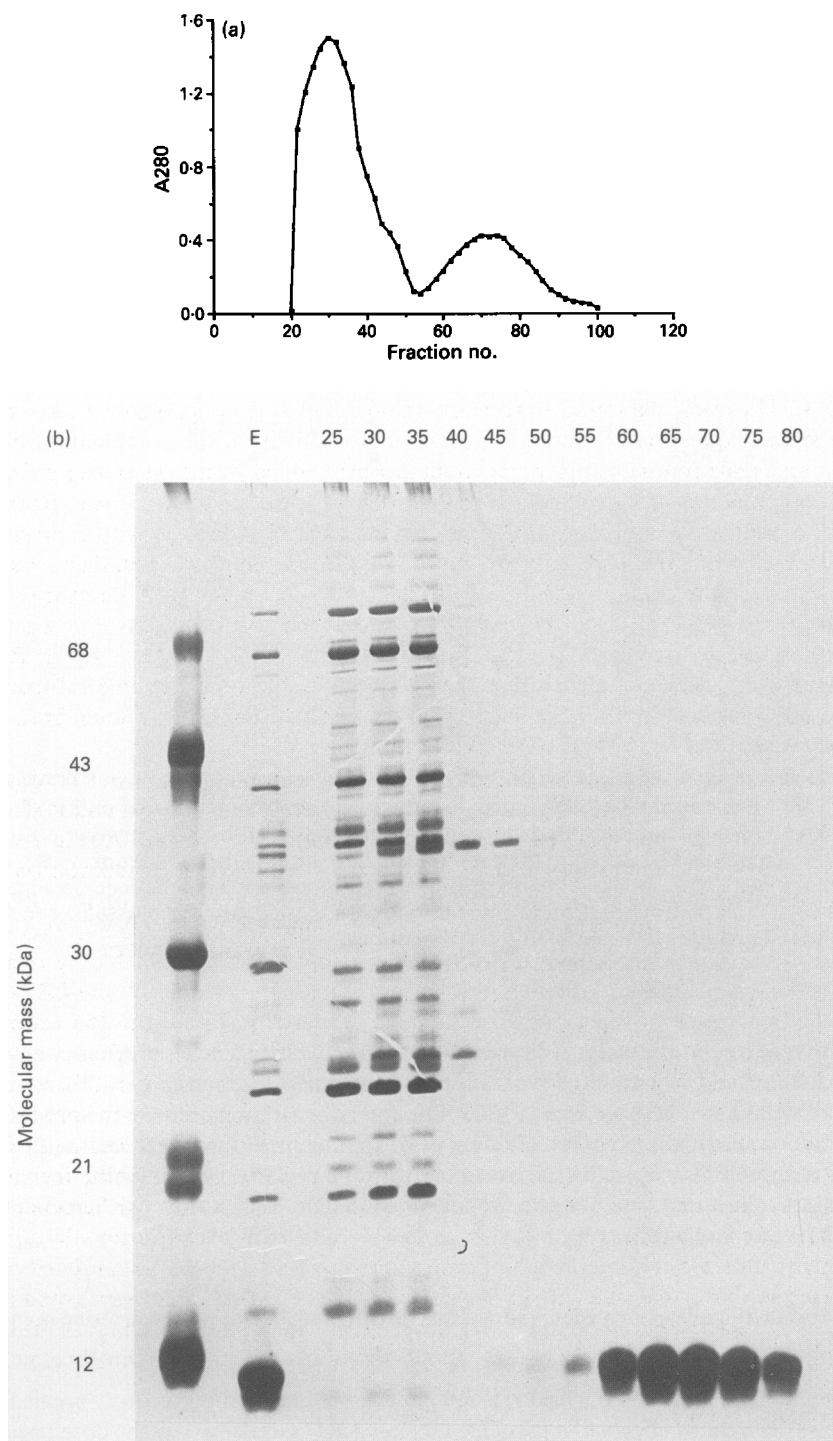


Fig. 3. Purification of the recombinant thioredoxin after cloning of the human thioredoxin coding sequence and expression of the wild type in *Escherichia coli*. (a) Purification of expressed thioredoxin in carboxymethyl (CM) cellulose chromatography and (b) SDS PAGE analysis of the cell extract (E) and selected fractions across the CM cellulose profile.

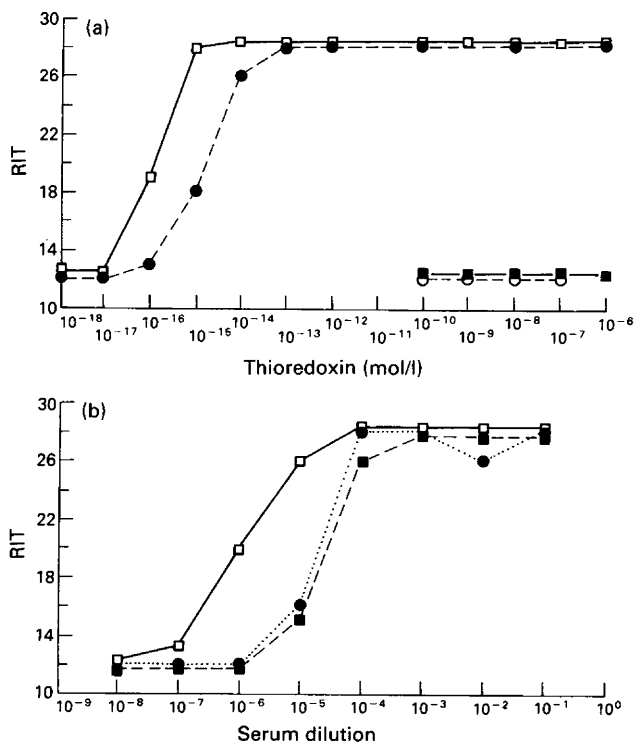


Fig. 4. Dose-response effects of (a) thioredoxin on the rosette inhibition titre (RIT) of mouse spleen cells when added alone (■), in combination with male mouse serum on simultaneous addition (□), in combination with male mouse serum but in a two-step incubation protocol in which the serum was first added to the cells followed by washing then thioredoxin (●), and in combination with male mouse serum in the two-step protocol but with thioredoxin added first (○); and (b) three male mouse sera in co-operating with thioredoxin (1nM) to induce increased RITs. All data points are means of 3–4 determinations.

cells in the rosette inhibition assay. All types of mice sera, male or female, pregnancy and nonpregnancy, had the capacity to induce the generation of an active S2 fraction (Fig. 5a, b), comparable to that induced by PAF (see Orozco *et al.*, 1990). The ability of sera to stimulate the production of an active S2 fraction was completely inhibited by prior treatment of the spleen cells with the lipoxigenase inhibitor NDGA but not by the treatment with the cyclooxygenase inhibitor indomethacin (Fig. 5c), again indicating that the production of an active S2 fraction was dependent upon a functional lipoxigenase pathway.

Thioredoxin is functionally equivalent to the oestrus-specific components of mouse serum in co-operating with a PAF stimulus

A combination of PAF and purified recombinant thioredoxin simultaneously applied to mouse spleen cells was extremely effective in inducing increased RITs as shown by the dose response (Fig. 6). Also, in two-step incubation protocols, increased RITs were observed only when PAF was added in the first step, followed by thioredoxin in the second (Fig. 6), indicating that thioredoxin was functionally equivalent to the oestrus-specific serum components (Orozco *et al.*, 1990) by acting to reverse the PAF-induced refractory state.

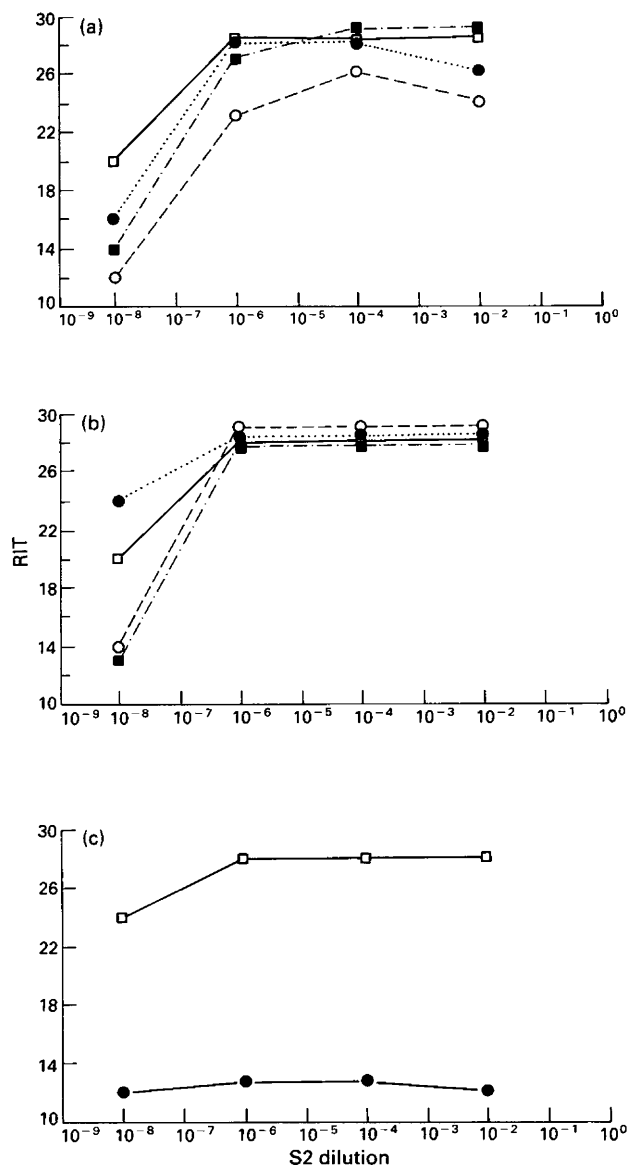


Fig. 5. Dose-response in the rosette inhibition assay of S2 fractions produced by stimulation of spleen cells (a) with 4 male mouse sera. (□, ■, ○, ●); (b) with 4 sera from pregnant mice; (□) Day 3, (○) Day 6, (■) Day 10 and (●) Day 14 pregnancy serum; and (c) male mouse serum after treatment of cells with $10\mu\text{M}$ indomethacin (□) or $20\mu\text{M}$ nordihydroguaiaretic acid (●). All data points are means of 3–4 determinations; RIT, rosette inhibition titre.

Relationship of the oestrus-dependent mouse serum components to thioredoxin

This functional equivalence of thioredoxin and the oestrous mouse serum in interacting with PAF-stimulated cells to allow for the expression of increased RITs suggested that the effective components in oestrous mouse serum may be related to thioredoxin. To test this possibility, oestrous mouse sera together with metoestrous, dioestrous and male mouse sera were adsorbed with solid-phase anti-thioredoxin (test) or pre-immune (control) gammaglobulins. The test and control adsorbed

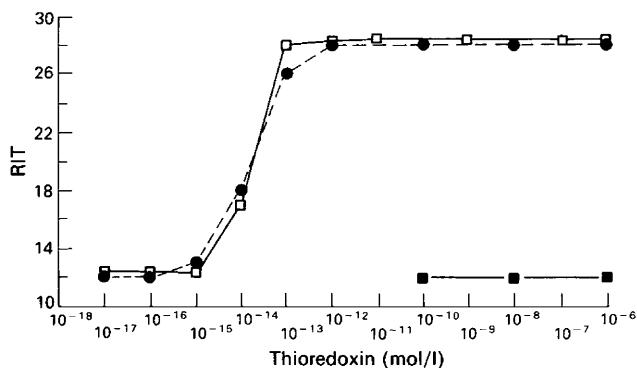


Fig. 6. Dose–response effects of thioredoxin on the rosette inhibition titre (RIT) when tested in combination with 5nM platelet-activating factor (PAF) when both were added simultaneously (□), when PAF was added first in a two-step incubation protocol (●) and when thioredoxin was added first in a two-step protocol (■). All data points are means of 3–4 determinations.

samples with the pH 2 eluates from these adsorptions were then tested for their ability to co-operate with PAF in inducing increased RITs. As shown by the dose–response data (Fig. 7), oestrous mouse serum (but no other non-pregnancy serum) could co-operate with PAF to induce increased RITs. This capacity was specifically removed by adsorption with anti-thioredoxin antibodies (Fig. 7a) and could be specifically recovered in the pH 2 eluates from such adsorptions, indicating that the components in oestrous mouse serum which act to reverse the PAF-induced refractory state were related to thioredoxin. None of the other non-pregnancy sera, their adsorbed supernatants, or pH 2 eluates (see Fig. 7a) possessed the capacity to co-operate with PAF in inducing increased RITs, again indicating that this capacity (due, at least in part, to components related to or associated with thioredoxin), was oestrus-dependent.

Involvement of thioredoxin-related proteins in the ‘EPF’ phenomenon

A final series of experiments was performed to demonstrate the direct involvement of thioredoxin-related proteins in the EPF phenomenon. As EPF activity has been defined as the ability of pregnancy sera to induce increased rosette inhibition titres in the rosette inhibition assay, we asked whether specific anti-thioredoxin antibodies could specifically remove this capacity from pregnancy sera. The preparation of recombinant human thioredoxin, free from contamination by other vertebrate proteins, was used to generate specific antisera. The gammaglobulin fractions were isolated from selected immune and pre-immune sera and coupled to Affi-Gel for use as solid-phase adsorbents. Extensive adsorption experiments performed with these reagents (see Fig. 7b) revealed that (i) the ability of pregnancy sera to induce the expression of increased RITs was lost on adsorption with anti-thioredoxin antibodies; (ii) this ability could be recovered in the pH 2 eluates from the anti-thioredoxin adsorptions of pregnancy serum samples, but not from such adsorptions of non-pregnancy sera; (iii) the ability of anti-thioredoxin-adsorbed pregnancy sera to induce increased RITs could be restored by the addition of pure recombinant thioredoxin; and (iv) the ability to induce increased RITs could also be conferred on anti-thioredoxin-adsorbed samples of non-pregnancy serum by addition of thioredoxin.

Results (iii) and (iv) indicated that anti-thioredoxin antibody treatment of pregnancy and non-pregnancy sera did not remove the capacity of these sera to stimulate the production of S2 factors or the ability to render the stimulated cell population refractory to the action of these compounds. Thus, activity expression with these adsorbed sera, as with normal sera (Fig. 4a), could be achieved

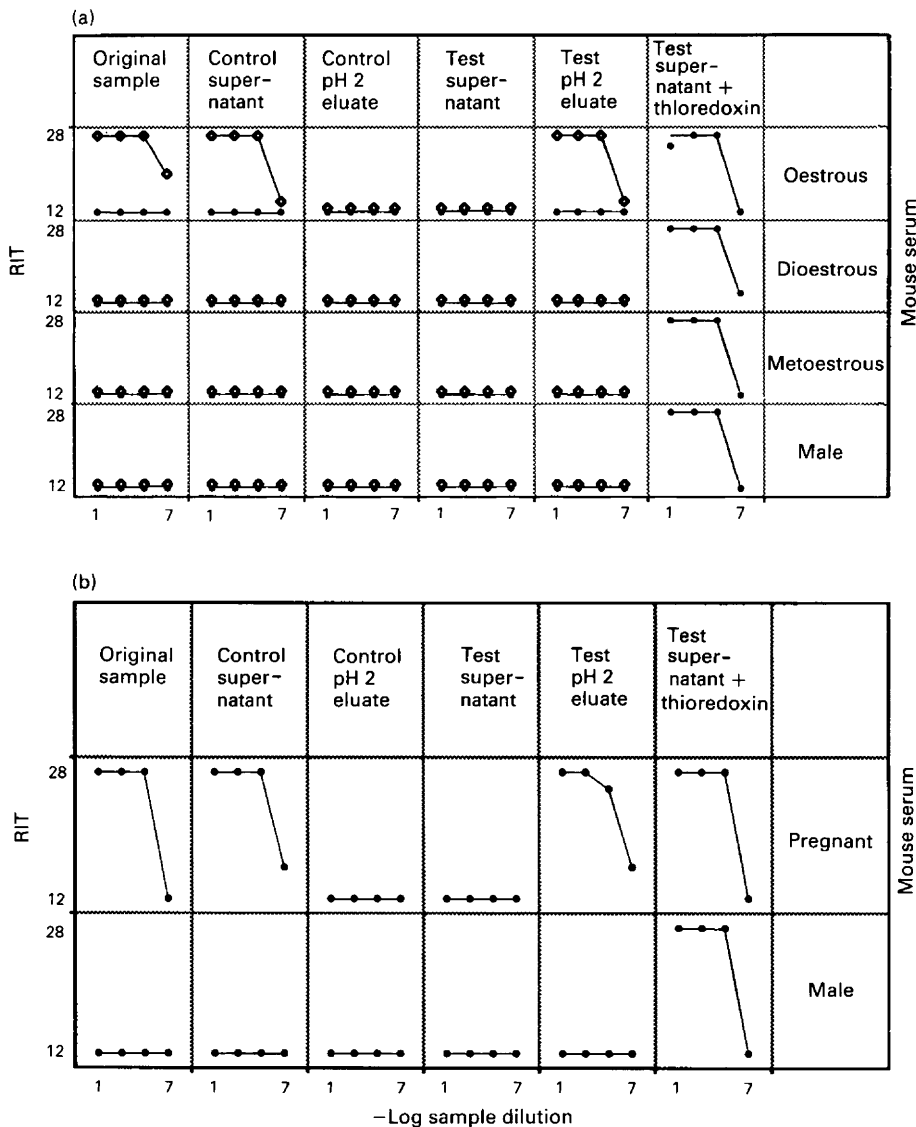


Fig. 7. Adsorption of (a) oestrous, dioestrous, metoestrous and male mouse sera and (b) pregnancy serum and male mouse serum with solid-phase anti-thioredoxin (test) or pre-immune (control) gammaglobulins. Rosette inhibition titre (RIT) activity dose-response profiles obtained on testing serial dilutions of the original sample, the sample after adsorption with control gammaglobulins (control supernatant), the pH 2 eluate from the control adsorption (control pH 2 eluate), the sample after adsorption with anti-thioredoxin antibodies (test supernatant), the pH 2 eluate from the anti-thioredoxin adsorption (test pH 2 eluate), and the test supernatant in combination with 1nM thioredoxin (test supernatant + thioredoxin). Data points are the means of duplicate or triplicate determinations and each experiment is representative of duplicate or triplicate experiments with each sample; results obtained on (●) testing sample alone, or (◇) in combination with 5nM platelet-activating factor.

by the addition of thioredoxin to the sample. The ability of these adsorbed sera to stimulate the production of an active S2 fraction could also be directly demonstrated (data not shown) as it was for non-adsorbed serum samples (Fig. 5a, b).

Discussion

These studies have identified thioredoxin as the major polypeptide component in our ovine placental preparation (Clarke *et al.*, 1987) which mimics the action of pregnancy sera by inducing the expression of increased rosette inhibition titres in the rosette inhibition assay. Pure thioredoxin produced by recombinant techniques was devoid of this activity, but could play a permissive role in this assay under certain circumstances. When pure thioredoxin was applied to spleen cells with or following appropriate cell stimuli such as PAF or sera, it resulted in an increased rosette inhibition titre where none was achieved in its absence. In general terms, it does this by preventing or reversing a refractory state induced in the spleen cells by these stimuli, allowing lipoxygenase-dependent products, also generated as a consequence of the stimulation, or naturally present in the sample in the case of serum, to exert their effects. The nature of the refractory state and the mechanistic detail of its reversal by thioredoxin are not known and are the subject of continued study. In these studies, site-directed mutagenesis techniques are being applied to examine whether the classical protein disulphide reductase activity of thioredoxin (Holmgren, 1979, 1989) is directly involved.

This is not the first report of isolated preparations having thioredoxin as the only or major polypeptide component and which display potent activity in biological assays. Tagaya *et al.* (1989) reported a thioredoxin-containing preparation (designated ADF) from adult T-cell leukaemia-derived cells with interleukin-2-receptor-inducing and growth stimulatory activities for human T cells. Rimsky *et al.* (1986) reported the isolation of a 12 kDa polypeptide preparation from the culture supernatants of an EBV-transformed B-cell line, which displayed interleukin-1-like activities in a range of biological assays (Wakasugi *et al.*, 1987); this polypeptide was later identified as thioredoxin (Wollman *et al.*, 1988). The expressed recombinant product had classical thioredoxin-like properties, but no data concerning its possible function in interleukin-1 biological assays were presented. We have found that purified recombinant thioredoxin alone is not active in the rosette inhibition assay, whereas thioredoxin preparations isolated from ovine placental extracts are. This, with the demonstration that the RIT-active principles could be partially resolved from the thioredoxin in the placental preparation, suggests that the activity of the preparation was due to molecules that may bind to thioredoxin and so co-purify with it. The identity of these molecules remains to be established; they may be arachidonic acid metabolites. Such an interpretation would be consistent with data presented elsewhere (Clarke *et al.*, 1990b) showing that metabolites of arachidonic acid, notably the leukotrienes, are active in the rosette inhibition assay. With respect to the thioredoxin preparations of others (Rimsky *et al.*, 1986; Wakasugi *et al.*, 1987) with interleukin-1-like activities, metabolites of the lipoxygenase pathway are increasingly recognized (Farrar & Humes, 1985; Goodwin & Behrens, 1988) as possible second messengers of interleukin-1 action and certain of these metabolites may substitute for interleukin-1 in most biological assays.

As discussed earlier, many studies have tried to define the molecular basis of EPF activity expression by pregnancy serum by attempting to isolate molecules active in the rosette inhibition assay, and many preparations have been reported (Wilson *et al.*, 1983; Cavanagh, 1984, 1985; Bose *et al.*, 1989; Mehta *et al.*, 1989) with the ability to induce increased rosette inhibition titres. These, however, differ remarkably in apparent molecular composition. Our present studies differ from these earlier reports, including that (Wilson *et al.*, 1983) from our own laboratory, in two fundamental aspects. Firstly, the polypeptide component of our preparation has been unequivocally identified as thioredoxin and a role for the pure protein in the rosette inhibition assay has been defined. Secondly, in no other case has it been documented that specific antibodies raised against an identified molecule remove from pregnancy sera the ability to induce the expression of increased rosette inhibition titres.

This documentation is critically important as it establishes the relevance of the isolated molecule to the mechanisms which allow pregnancy sera to induce increased RITs, i.e. to express so-called EPF activity. As we have seen in this and other recent studies (Clarke *et al.*, 1990a, b; Orozco *et al.*,

1990), there are now a number of individual molecules (arachidonic acid and the leukotrienes B₄, C₄, D₄ and E₄, for example) or combinations of molecules (PAF plus thioredoxin, PAF plus oestrus-specific components of mouse sera, and sera plus thioredoxin, for example) which can be shown to induce increased RITs. No doubt there are others. The critical question is which molecule or combination of molecules present in pregnancy serum is responsible for its ability to induce increased RITs. The present results provide an initial answer that thioredoxin-like molecules and molecules directly or indirectly associated with them are involved and so a new concept of the EPF phenomenon emerges, which in many respects is the antithesis of that which has guided many studies in this field. The common usage of the term EPF has implied the existence in pregnancy serum of a single, unique factor, which has usually been assumed to be a novel, pregnancy-specific protein with the inherent capacity to induce increased RITs. (Cavanagh, 1985; Morton *et al.*, 1987; Bose *et al.*, 1989; Mehta *et al.*, 1989). This concept is rendered untenable by the present results.

In seeking to gain an appreciation of the real nature of the EPF phenomenon, it is just as pertinent to ask why non-pregnancy sera do not induce increased RITs as it is to ask why pregnancy sera do. This is so because recent studies (Clarke *et al.* 1990b) have revealed that many molecules (arachidonic acid and the leukotrienes, for example) which are normal serum constituents (Beaubien *et al.*, 1984; Hughes *et al.*, 1989) can, in isolation, induce the expression of increased RITs. Moreover, in the present studies we found that all sera can stimulate mouse spleen cells to produce further active moieties (the S2 factors) which have the capacity to induce increased RITs when applied to fresh, non-stimulated spleen cells. Thus, non-pregnancy sera possess not only endogenous active moieties, but also the capacity to stimulate the production of more during the assay procedure, yet they fail to induce increased RITs. This is so because these sera also induce a refractory state preventing the endogenous and induced active moieties from exerting their effects. This refractory state could be induced and maintained by any of a variety of potential stimuli and inhibitory or counteracting substances to be found in sera. Precedence for cell stimuli inducing a refractory state has been provided (Orozco *et al.*, 1990) by the demonstration that PAF or calcium ionophore stimulation of mouse spleen cells renders them refractory to the action of the active S2 factors, and specific examples of potential counteracting substances have been provided by the demonstration (Clarke *et al.*, 1990b) that certain prostaglandins may counteract the action of leukotrienes in inducing increased RITs. While the specific serum moieties responsible for the refractory state remain to be identified, the induction of it is a matter of experimental record, as is its reversal by thioredoxin. Thioredoxin is itself, of course, a widely distributed protein (Rozell *et al.*, 1985) and would also be expected to be a constituent of normal serum (Holmgren & Luthman, 1978; F. M. Clarke, unpublished). The observed failure of non-pregnancy sera to induce increased RITs implies that the thioredoxin or thioredoxin-related molecules in these sera are in a form, or are rendered to a form, incapable of reversing the refractory state.

Pregnancy sera, like all other sera, would also be expected to contain RIT active moieties, such as the leukotrienes, and they also possess the capacity to induce S2 factor production during the assay procedure, but clearly the refractory state, a consequence of stimulation by all other sera, is either not induced or is rapidly reversed, because cells treated with pregnancy sera do display increased RITs. It is unlikely that pregnancy sera lack the constituents which normally would induce the refractory state. The failure of the anti-thioredoxin-adsorbed pregnancy sera to induce increased RITs and the restoration of this capacity to these adsorbed sera by addition of recombinant thioredoxin (Fig. 7b) supports the contention that these constituents are indeed present. The observation of increased RITs with pregnancy sera then may imply the presence in these sera of components functionally equivalent to recombinant thioredoxin which reverse or prevent the refractory state. Thus, a distinguishing feature of pregnancy sera could be the presence of functional forms of thioredoxin-related molecules which act in a permissive role in concert with endogenous or induced active moieties to allow the expression of an increased RIT. All other sera may possess non-functional or inhibitable forms of these proteins and so fail to express increased RITs.

The results also suggest that there may be an association between the thioredoxin-related molecules of pregnancy sera and certain of the RIT active moieties in these sera. This possibility arises from considering data from the anti-thioredoxin adsorption experiments (Fig. 7b), which show that the pH 2 eluates from the solid-phase anti-thioredoxin adsorptions of pregnancy (but not non-pregnancy) sera had the capacity to induce increased RITs. As pure recombinant thioredoxin alone does not display this capacity, one explanation is that the anti-thioredoxin antibodies may have removed certain RIT active moieties which are directly or indirectly associated with the thioredoxin-like proteins of pregnancy sera, just as the isolation protocols applied to placental extracts resulted in the co-isolation of thioredoxin and active moieties. If such an association does exist it may distinguish pregnancy from non-pregnancy sera and may provide the basis for co-operative interactions which allow for the presence of functional thioredoxin-like molecules in pregnancy sera, which in turn allow the active moieties in these sera to express their effects in the presence of the otherwise counteracting substances to be found in all sera.

Among the non-pregnancy sera it is obvious that sera obtained from animals in oestrus must represent a special case. While such sera by themselves do not induce the expression of increased RITs, they alone can co-operate with a PAF stimulus to allow for increased RITs (Orozco *et al.*, 1990). In the context of the foregoing discussion, this suggests the presence in these sera of additional or alternative oestrus-specific components which interact with PAF-stimulated, but not serum- or ionophore-stimulated cells (see Clarke *et al.*, 1990a), to allow the effective generation in the assay of functionally active, thioredoxin-like molecules which reverse the PAF-induced refractory state. How this is achieved and how many oestrus-dependent components are involved is not known and certainly no specific mechanism is implied by use of the term 'effective generation'. It could be achieved by some form of activation, by protection against inactivation or by other means. Definition of the molecular mechanism is seen as particularly important as it should go a long way to explaining how the release of embryo-derived PAF *in vivo* allows for the rapid appearance in maternal serum of the so-called EPF activity so soon after fertilization.

These and other recent studies (Clarke *et al.*, 1990a, b; Orozco *et al.*, 1990) have begun to identify the molecules and mechanisms at work in the rosette inhibition assay which cause the expression of increased RITs. They have provided new insights into the molecules and mechanisms which allow pregnancy sera to achieve this effect. In so doing, they offer a new model for the EPF phenomenon, which we believe should now assist in the design of further experiments to resolve the many enigmatic properties previously ascribed to the so-called 'early pregnancy factor'.

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