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Identification of cyclophilin as the erythrocyte ciclosporin-binding protein

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Previous studies on the distribution of circulating ciclosporin have shown that the majority of the drug is associated with erythrocytes. In order to investigate the nature of ciclosporin-erythrocyte binding, binding studies were performed on isolated erythrocytes. At therapeutic concentrations (approx. 0.5 μg/ml in whole blood) > 90% of the erythrocyte associated ciclosporin was found in the cytosol. The cytosolic binding capacity was approximately (2–2.5) · 10^5 molecules of ciclosporin per cell. A lower affinity binding of the drug to the plasma membrane occurred only at higher ciclosporin concentrations. The ciclosporin-binding species was purified from erythrocyte cytosol using ciclosporin-Affigel affinity chromatography. This revealed a 16 kDa protein, similar in size to the ciclosporin-binding protein, cyclophilin, previously identified in lymphocyte cytosol. Immunochemical analysis using rabbit anti-bovine spleen cyclophilin antisera revealed that the erythrocyte ciclosporin-binding protein was either cyclophilin or a closely related protein. It is concluded that intracellular ciclosporin-binding within erythrocytes is mostly attributable to the presence of a single protein or protein family represented by cyclophilin. The presence of (2–2.5) · 10^5 copies of this binding protein within each erythrocyte is responsible for the ciclosporin found associated with erythrocytes.

Introduction

Ciclosporin, a cyclic undecapeptide of fungal origin, is now widely used for the prevention of graft rejection. The drug appears to act primarily on T-lymphocytes by inhibiting the transcription of mRNA for interleukin 2 and other lymphokines [1,2]. Although the detailed mechanism by which ciclosporin achieves this effect is unknown, a cytosolic 16–17 kDa polypeptide, cyclophilin, was recently identified as the intracellular receptor for ciclosporin in lymphocytes [3].

Clinical use of ciclosporin requires careful monitoring of circulating drug levels as the therapeutic window is very narrow [4–8]. In circulation, ciclosporin binds preferentially to the cellular compartment; at concentrations of 50–1000 ng ciclosporin per ml whole blood more than 70% of the drug is bound to erythrocytes [9].

The erythrocyte-plasma distribution is greatly affected by temperature with the erythrocytic ciclosporin pool increasing as the temperature is lowered [10,11]. Because of this influence on the distribution of ciclosporin between cellular and plasma compartments, therapeutic monitoring of the drug should be performed on whole blood [5,12].

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Because of the importance of monitoring circulating ciclosporin levels two recent studies have attempted to identify the erythrocyte ciclosporin-binding protein. The result of the first study [9] suggested that haemoglobin may act as the ciclosporin receptor in erythrocytes. However, a more recent and extensive study [13], has identified the erythrocyte ciclosporin-binding protein to be a 15–17 kDa polypeptide.

The present study extends previous work on ciclosporin–erythrocyte interactions and demonstrates that this binding consists of two components. A higher affinity saturable cytosolic binding which can accomodate $2.5 \cdot 10^5$ molecules of ciclosporin per cell. The other is a lower affinity membrane binding which occurs at higher ciclosporin concentrations. Using ciclosporin-Affigel affinity chromatography a 16 kDa ciclosporin-binding protein was also identified within erythrocyte, which has the same electrophoretic mobility as bovine spleen cyclophilin. Furthermore, this protein was found to be immunologically cross-reactive with bovine cyclophilin when Western-blotted and immunoprobed using rabbit anti-bovine cyclophilin antisera.

Materials and Methods

Rabbit anti-bovine thymus cyclophilin antiserum was a gift of Dr. M. Harding (Yale University, New Haven, CT, U.S.A.). [^{3}H]Ciclosporin (9 μCi/μg) was obtained from Dr. Voges (Sandoz Ltd., Basel, Switzerland). Soluble ciclosporin is [D-Lys^8]-ciclosporin. Ciclosporin-Affigel was produced by linking soluble ciclosporin via the lysine's amino group to Affigel (Bio-Rad, U.S.A.) by following the manufacturers instructions at a final concentration of 2 mg per ml of gel. Ciclosporin-Affigel was diluted 1 : 20 with ethanolamine-blocked Affigel and then washed with 1% bovine serum albumin followed by extensive washing with phosphate-buffered saline before use. Biotinylated donkey anti-rabbit antibody and biotinylated horseradish peroxidase-streptavidin complex were obtained from Amersham International (U.K.). Bovine serum albumin, human haemoglobin and vitamin-free casein were purchased from Serva (F.R.G.). Human serum albumin, 3,3-diaminobenzidine, phenylmethylsulphonyl fluoride (PMSF) and another human haemoglobin preparation were obtained from Sigma. Sephadex LH-20 was obtained from Pharmacia (Sweden).

Cell and lysate preparation

Human blood was collected by venipuncture into heparinized tubes. The cells were pelleted by centrifugation at $2500 \times g$ for 10 min, after which the plasma and buffy layer were removed, and then washed three times in phosphate-buffered saline and used on the same day. For lysate preparation the erythrocytes were washed once in ice cold 0.1 M sodium phosphate (pH 8.0) and lysed in 1.5 volume of 5 mM sodium phosphate, (pH 8.0). The lysate was centrifuged at $40\,000 \times g$ at 4 °C for 30 min followed by $100\,000 \times g$ for 3 h to remove plasma membranes and other particulate matter. The lysate was dialysed against 200 volumes of phosphate-buffered saline at 4 °C, overnight (three times). The dialysed lysate containing sodium azide (0.02%) and PMSF (1 mM) was stored at −20 °C.

Binding studies

To prevent interference from plasma proteins all the following studies were performed on washed erythrocytes suspended in phosphate-buffered saline containing 1% bovine serum albumin.

Binding studies with increasing ciclosporin concentrations. Erythrocytes were suspended in 1 ml at an hematocrit of 50% and [^{3}H]ciclosporin from 0.5 ng to 16 μg/ml (final concentration) added. The suspensions were mixed and left at room temperature for 1 h. The cells were then pelleted by centrifugation at $9000 \times g$ for 30 s and supernatant was removed after which the cells were washed three times with ice-cold phosphate-buffered saline. Samples for counting were added to 20 ml scintillation vials containing 500 μl of 30% H_2O_2 to decolorise the sample. The samples were counted with 20 ml of Lumagel using a Packard Tricarb-2000 CA. This procedure was used in all the following studies.

Binding studies with decreasing cell density. Washed erythrocytes were suspended in 1 ml phosphate-buffered saline containing 1% bovine serum albumin at varying cell densities: from $2.4 \cdot 10^9$ to $1.2 \cdot 10^6$ cells/ml (25% hematocrit/0.01% hematocrit). [^{3}H]Ciclosporin (0.5 μg/ml) was ad-

ded and the cells were incubated for 1 h at room temperature and then processed as above.

Studies on the distribution of [^{3}H]ciclosporin between cytosolic and membrane compartments were performed as above except the washed cells were lysed by sonication for 15 s followed by rapid freezing on dry ice and quick thawing. The membranes were pelleted by centrifugation at $14000 \times g$ for 15 min at 4°C. The membranes were washed extensively with cold 5 mM sodium phosphate to remove all visible haemoglobin and suspended in 50% methanol before counting of the radioactivity.

Erythrocyte uptake of [^{3}H]ciclosporin in the presence of competing proteins

Washed erythrocytes were suspended at 50% hematocrit in the following protein containing solutions: bovine serum albumin, human serum albumin, haemoglobin, human plasma and erythrocyte lysate (0–70 mg protein/ml). All the preparations were dissolved and/or diluted in phosphate-buffered saline. The cell suspensions were incubated with [^{3}H]ciclosporin (1 μg/ml) for 1 h at room temperature with occasional agitation. Sampling and counting of the cells and supernatant was as previously described.

Extraction of lysate and haemoglobin preparations with ciclosporin-Affigel

Haemoglobin was dissolved in phosphate-buffered saline at a concentration of 70 mg/ml, approximately the protein concentration of the erythrocyte lysate (75 mg/ml). The solutions were centrifuged at $15000 \times g$ for 15 min at 4°C to remove any insoluble material after which PMSF (1 mM) was added. 100 μl of 50% (v/v) suspensions of Affigel or ciclosporin-Affigel were then added to 500 μl samples of the lysate or the haemoglobin solutions followed by incubation for 3 h at 4°C with constant agitation. The gel matrices were pelleted by a brief centrifugation and the supernatant removed and stored at 4°C. The gels were washed six times with 1 ml phosphate-buffered saline containing 5 mM 2-mercaptoethanol and 0.1% (v/v) Tween 20 followed by suspension at 50% (v/v) in phosphate-buffered saline containing 5 mM 2-mercaptoethanol. A 30 μl sample was removed for SDS-PAGE [14] analysis (see below) and to the remainder, 100 μl of a solution containing soluble ciclosporin (2 mg/ml) in 20 mM sodium phosphate (pH 6.2) was added. The sample was left incubating overnight at 4°C. The Affigels were pelleted, the supernatants were removed, and then the gels were washed twice with 200 μl phosphate-buffered saline containing 5 mM 2-mercaptoethanol. The original supernatant and the first wash supernatant were pooled and then concentrated (to 100 μl) using a Spedivac concentrator. Analysis of samples by sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as previously described [14]. Samples were normally equally divided between identical 15% polyacrylamide gels. Gels were either silver stained for protein using the Bio-Rad Silver Staining kit or the proteins were electrophoretically transferred to nitrocellulose (4 h at 120 V using a Bio-Rad Trans-Blot Cell) for immunoblotting. The nitrocellulose paper was washed overnight at 4°C in phosphate-buffered saline containing 1% Nonidet P-40 after which the paper was immunoperoxidase stained by incubation in the following phosphate-buffered saline solutions:

(1) Incubation for 1 h at 37°C in 1% (w/v) casein containing 0.04% (w/v) sodium azide;
(2) 3 washes in 0.25% (v/v) Tween 20;
(3) Incubation at room temperature for 2 h in 1% (w/v) casein containing 0.04% sodium azide and (1 : 2000) rabbit anti-bovine cyclophilin antibody;
(4) 5 washes in 0.25% (v/v) Tween 20;
(5) Incubation at room temperature for 1 h in casein/azide containing (1 : 250) biotinylated donkey anti-rabbit antibody;
(6) 6 washes in 0.25% (v/v) Tween 20;
(7) 30 min incubation at room temperature with (1 : 1000) biotinylated peroxidase-streptavidin complex in phosphate-buffered saline containing 1% bovine serum albumin;
(8) 5 washes with 0.25% (v/v) Tween 20;
(9) Addition of substrate solution consisting of 0.5 mg/ml 3,3-diaminobenzidine, 0.3 mg/ml cobalt chloride and (10 μl/50 ml) 30% vol. H_2O_2;
(10) The reaction was stopped by extensive washing in distilled water.

Sephadex LH-20 column assay of ciclosporin-binding activity

This assay was performed essentially as previously described [3]. Microcolumns (2.0 ml) of Sephadex LH-20 resin were preequilibrated in Tris-HCl buffer (20 mM, pH 7.2) containing 2-mercaptoethanol (5 mM) and sodium azide (0.02%). 30 µl samples of lysate or haemoglobin solution were diluted to 90 µl with Tris buffer in Eppendorf tubes followed by the addition of 10 µl [^{3}H]ciclosporin ($3 \cdot 10^{-7}$ M, 0.5 µCi/ml) in 40% ethanol. The tube contents were mixed and incubated at 37°C for 15 min; 50 µl was applied to the column, and 25 µl was assayed for radioactivity in 10 ml of Lumagel. Columns were eluted with the above buffer and the excluded volume collected and assayed for radioactivity.

Purification of bovine spleen cyclophilin

Bovine spleen cyclophilin was purified essentially as described previously [3]. Calf spleen was homogenised in 1 kg portions, centrifuged at $20\,000 \times g$ for 30 min and the supernatant fractionated on an Amicon Hollowfiber ultrafiltration system. The < 100 kDa fraction was concentrated on a 5 kDa exclusion membrane and applied to a 2.5 × 30 cm column containing Amicon Matrix Gel blue-A resin. The column was washed with 20 mM potassium phosphate buffer (pH 7.2) and the cyclophilin containing material was eluted with 100 mM potassium phosphate (pH 7.2). The eluate was concentrated, equilibrated with 5 mM glycine buffer (pH 5.7) and loaded onto a sucrose gradient containing Ampholines between pH 3 and 11 in a LKB 440 ml focusing column. Isoelectric focusing was performed for 48 h at 6°C at a constant 5 W (starting at 230 V, ending at 680 V). The cyclophilin fraction was eluted from the column, immediately adjusted to neutral pH and dialysed against 5 mM glycine before refocusing on a 110 ml column at pH 8–11. The cyclophilin containing fraction was concentrated, dialysed against the CM-column equilibration buffer and applied to a HPLC carboxymethyl column (LKB Ultropac TSK CM-3SW, 21.5 × 250 mm). The column was equilibrated with 5 mM potassium phosphate (pH 7.2) containing 5 mM 2-mercaptoethanol and 0.02% sodium azide. Cyclophilin was eluted as a double peak using a linear gradient of sodium chloride (0 M to 0.5 M) in equilibration buffer.

Results

The binding, and subcellular distribution of ciclosporin in erythrocytes

Radiolabelled ciclosporin binding to erythrocytes in phosphate-buffered saline containing 1% bovine serum albumin was saturated at a concentration of 3 µg/ml (Fig. 1) when $(2–2.5) \cdot 10^5$ molecules of ciclosporin were bound per cell. When the ciclosporin concentration was increased to 16 µg/ml a second lower affinity binding was observed, increasing the number molecules binding per cell to $5.2 \cdot 10^5$.

To characterise this second binding phenomenon, even though solubility limits further concentration increases, binding was examined at fixed ciclosporin concentration but decreasing cell density (Fig. 2). At the highest cell density of $2.4 \cdot 10^9$ cells/ml, approximately $7.5 \cdot 10^4$ molecules of ciclosporin were associated with each erythrocyte. When this cell density was halved ciclosporin-binding almost doubled to $14 \cdot 10^4$ molecules per cell. Further dilutions of cell density increased ciclosporin binding little so that by the sixth dilution point ($7.5 \cdot 10^7$ cells/ml) the number of mole-

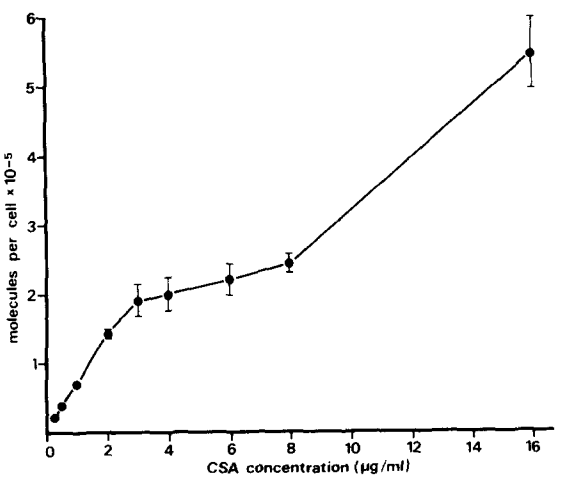

Fig. 1. Erythrocyte uptake of [^{3}H]ciclosporin in the presence of increasing concentrations of [^{3}H]ciclosporin (CSA). Performed as described in Materials and Methods. The data represents the mean ± S.D. ($n = 6$).

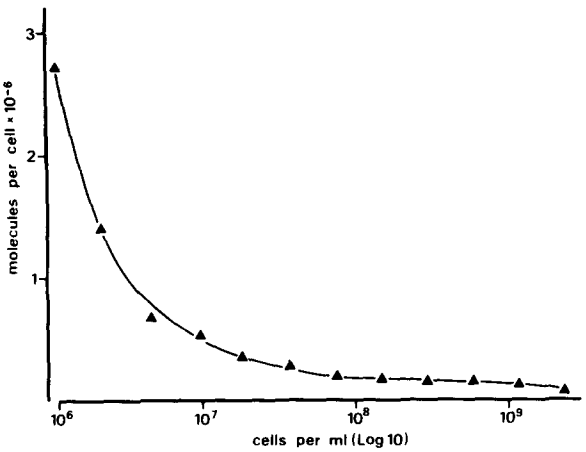

Fig. 2. Effect of increasing cell density on the uptake of [^{3}H]ciclosporin by erythrocytes. Performed as described in Materials and Methods. Concentration of [^{3}H]ciclosporin 0.5 μg/ml. The data represents the mean (S.D. were within 15% of the mean value) ($n = 6$).

cules per cell had increased only to $22 \cdot 10^4$. Further reductions in cell density, especially below 10^7 cells/ml, increased ciclosporin-binding with the cells. At the lowest erythrocyte density examined of $1.2 \cdot 10^6$ cells/ml, $2.7 \cdot 10^6$ ciclosporin molecules were bound to each cell with no evidence of saturation having been reached.

When the distribution of ciclosporin in membrane and cytosol was analysed over the same range of cell densities (Fig. 3), ciclosporin in the cytosol was found to increase as the cell concentration was was decreased until a saturation level of $2.5 \cdot 10^5$ molecules per cell cytosol was reached at a cell density of approximately 10^8 cells/ml. Conversely, only very low amounts of ciclosporin, less than 5000 molecules, were found associated with the cell membrane at these higher cell concentrations. However, reduction of cell number below 10^8 cells/ml resulted in a large increase in ciclosporin binding to the membrane. This binding which was apparently nonsaturable, reached $2 \cdot 10^6$ molecules per cell membrane at the lowest cell density tested. Summation of the membrane and cytosolic ciclosporin-binding components at each cell density was found to reproduce the ciclosporin-binding curve recorded in the previous study (Fig. 2). Also, the triphasic ciclosporin-erythrocyte binding obtained in Fig. 1 was again observed.

Inhibition of erythrocyte [^{3}H]ciclosporin uptake by the presence of competing protein solutions

The uptake of [^{3}H]ciclosporin by erythrocytes in presence of various protein containing solutions is shown in Fig. 4. Both serum albumins had no

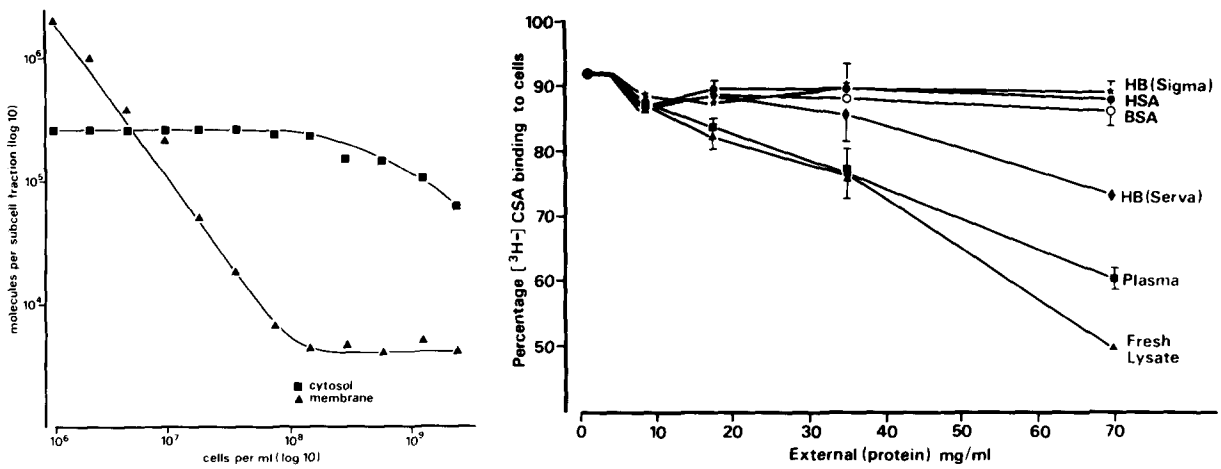

Fig. 3. Effects of increasing cell density on the subcellular distribution of [^{3}H]ciclosporin. Performed as described in Materials and Methods. Concentration of [^{3}H]ciclosporin 0.5 μg/ml. Each data point represents the mean (S.D. were within 15% of the mean value) ($n = 3$).

Fig. 4. Inhibition of erythrocyte [^{3}H]ciclosporin ([^{3}H]CSA) uptake by the presence of competing protein solutions. [^{3}H]Ciclosporin concentration was 1 μg/ml. The data represents the mean $\pm$ S.D. ($n = 6$). Key: Hb, haemoglobin; HSA, human serum albumin; BSA, bovine serum albumin.

TABLE I

INHIBITION BY NON-RADIOACTIVE CICLOSPORIN OF [^{3}H]CICLOSPORIN BINDING TO ERYTHROCYTES

Washed erythrocytes were suspended at 50% hematocrit in phosphate buffered saline and ciclosporin (at indicated concentrations) and [^{3}H]ciclosporin (1 μg/ml) added followed by incubation for 1 h at room temperature. Sampling and counting of cells was as described in Materials and Methods.

Ciclosporin (μg/ml)	Percentage [^{3}H]ciclosporin (1 μg/ml) binding to cells (±S.D., $n = 6$)
0	94% (±6)
1	69% (±6)
10	17% (±3)
25	11% (±1)

measurable ciclosporin-binding capacity as assessed by the failure of these solutions to prevent the uptake of ciclosporin by erythrocytes. Plasma, as expected, competed with erythrocytes for ciclosporin and at 70 mg/ml reduced erythrocyte uptake of ciclosporin by a third. Sigma haemoglobin showed little binding activity but Serva haemoglobin at the highest protein concentration reduced erythrocytic ciclosporin uptake by approximately one-sixth. Most effective was the fresh erythrocyte lysate preparation which at 70 mg/ml reduced the ciclosporin-binding to the erythrocytes by nearly one half. As a comparison Table I shows the inhibition of erythrocyte [^{3}H]ciclosporin uptake in the presence of competing non-radioactive ciclosporin which demonstrates that the binding of [^{3}H]ciclosporin to erythrocytes is specific with 80% of the radioactive ligand binding being displaced at a ciclosporin concentration of 10 μg/ml.

SDS-polyacrylamide analysis of proteins binding to ciclosporin-Affigel

Samples of fresh erythrocyte lysate and solutions of Serva and Sigma haemoglobin were extracted with ciclosporin-Affigel or uncoupled Affigel. The matrices were extensively washed followed by elution of specifically bound proteins with a water-soluble ciclosporin analogue.

Analysis of the proteins binding to ciclosporin-Affigel revealed the presence of a single specifically bound protein of approximately 16 kDa (Fig.

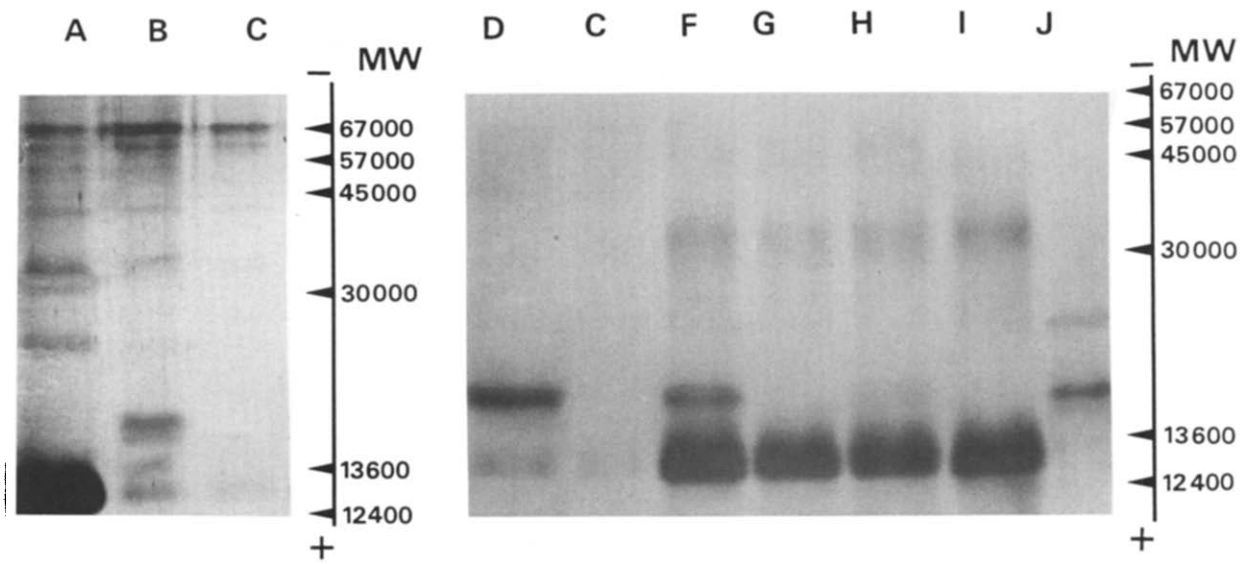

Fig. 5. SDS-PAGE analysis of erythrocyte proteins binding to, and eluting from, ciclosporin-Affigel. Key: human erythrocyte lysate (30 μg protein) (track A); human erythrocyte lysate proteins bound to ciclosporin-Affigel (track B); human erythrocyte lysate proteins bound to Affigel (track C); proteins eluted with soluble-ciclosporin from B (track D); proteins eluted with soluble ciclosporin from C (track E); Serva haemoglobin preparation proteins eluted with soluble ciclosporin from ciclosporin-Affigel (track F); as F except using Affigel (track G); Sigma haemoglobin preparation proteins eluted with soluble ciclosporin from ciclosporin-Affigel (track H); as H except using Affigel (track I); semipure bovine spleen cyclophilin (250 ng) (track J).

5, track B). This protein was not detectable in the original erythrocyte cell lysate (Fig. 5, track A). Other proteins were detected which bound to ciclosporin-Affigel, however, these were apparently non-specific since they also bound to the Affigel control (Fig. 5, track C). The same 16 kDa protein was also extracted from the Serva hemoglobin preparation although at lower levels as assessed by staining intensity. Only non-specifically bound proteins were obtained from the Sigma preparation (results not shown).

Analysis of proteins eluted from the matrices, using the soluble ciclosporin analogue, confirmed the above findings. The 16 kDa lysate polypeptide was the only protein eluted from the ciclosporin-Affigel by soluble ciclosporin, with exception of non-specifically bound haemoglobin (Fig. 5, track D). The same result was obtained in the Serva haemoglobin preparation (Fig. 5, track F) except that there was greater contamination from other non-specific proteins and the amount of the 16 kDa protein extracted was one half that extracted from the lysate (assessed by densitometric scanning of the gel). The 16 kDa protein was also extracted from the Sigma preparation, although at very low levels.

The electrophoretic mobility of the erythrocyte ciclosporin-binding protein was identical to that of bovine spleen cyclophilin (major band, Fig. 5, track J). To investigate whether the erythrocyte ciclosporin-protein was cyclophilin or a closely related protein Western-blotting followed by immunoperoxidase-staining with rabbit anti-bovine cyclophilin was performed (Fig. 6). As shown, both the lysate and Serva 16 kDa ciclosporin-binding proteins were recognized by rabbit anti-bovine cyclophilin (Fig. 6, tracks A and C) as was bovine cyclophilin (Fig. 6, track G). The Sigma protein gave a visible but weak signal (Fig. 6, track E, not reproducible on the photograph). This strongly suggests that the erythrocyte binding protein is cyclophilin or a closely related human version thereof.

LH-Sephadex column assay of lysate and haemoglobin samples

To further test the relationship between the identified 16 kDa human erythrocyte 'cyclophilin' and the ciclosporin-binding activity of the investigated lysate and haemoglobin samples, the preparations were analysed for ciclosporin-binding activity using the LH-column assay [3]. In this assay ciclosporin-binding function is measured by the amount of [^{3}H]ciclosporin appearing in the LH-column's excluded volume eluting with the

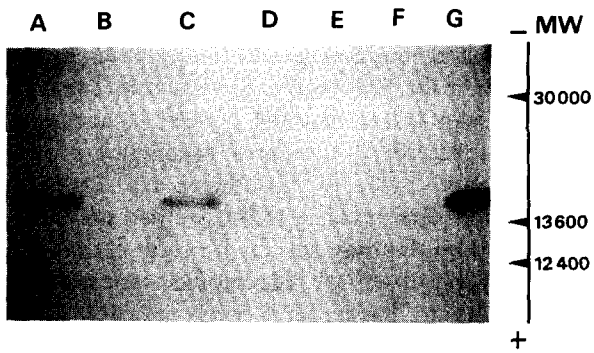

Fig. 6. Western blot and immunoperoxidase staining of erythrocyte ciclosporin-binding proteins with rabbit anti-bovine cyclophilin. Key: Human erythrocyte lysate proteins eluted from ciclosporin-Affigel (track A) or Affigel (track B) with soluble ciclosporin; Serva haemoglobin preparation proteins eluted from ciclosporin-Affigel (track C) and Affigel (track D) with soluble ciclosporin; Sigma haemoglobin preparation proteins eluted from ciclosporin-Affigel (track E) and Affigel (track F) with soluble ciclosporin; semi-pure bovine spleen cyclophilin (250 ng) (track G).

TABLE II

THE EFFECT OF CICLOSPORIN-AFFIGEL PREABSORPTION ON THE CICLOSPORIN-BINDING ACTIVITY OF LYSATE AND HAEMOGLOBIN PREPARATIONS AS MEASURED BY THE SEPHADEX LH-20 ASSAY

Data represent the percentage of [^{3}H]ciclosporin loaded on the Sephadex LH-20 column which was eluted with the protein in the excluded volume. Each measurement represents the average of three assays (±S.D.). n.d., not determined. BSA, bovine serum albumin.

Solution	Unabsorbed	Affigel absorbed	Ciclosporin-Affigel absorbed
Human erythrocyte lysate	38% (±2)	40% (±3)	7% (±2)
Serva haemoglobin	24% (±3)	26% (±1)	12% (±4)
Sigma haemoglobin	15% (±3)	15% (±2)	7% (±2)
BSA	9% (±3)	n.d.	n.d.

protein. The elution of free [^{3}H]ciclosporin from the column is retarded due to hydrophobic interactions with the LH-matrix. As shown in Table II fresh human lysate had the highest ciclosporin-binding followed by the Serva haemoglobin preparation and lastly by the Sigma haemoglobin preparation. Extraction with Affigel had no effect on ciclosporin-binding activity however extraction with ciclosporin-Affigel reduced ciclosporin-binding activity to background levels in each case. These results are in agreement with ciclosporin-binding activities measured in erythrocyte competition assay (Fig. 4) and the observation that the extracted 16 kDa protein is the erythrocyte cytosolic ciclosporin-binding protein.

Discussion

Taken together all the data presented in Figs. 1–3 showed that ciclosporin binding to erythrocytes consists of both cytosolic and membrane components. The cytosolic component has the higher affinity and is saturable at $(2–2.5) \cdot 10^5$ molecules of ciclosporin bound per cell. The binding of ciclosporin to the erythrocyte membrane remains at very low levels (< 5000 moles per cell membrane) until the cytosolic component is saturated after which large scale binding of ciclosporin to the membrane occurs. At the highest level of ciclosporin-membrane binding detected, $2 \cdot 10^6$ molecules per cell, there was no evidence of saturation having been reached and the total binding capacity of the membrane may be much higher. The high level of ciclosporin-membrane binding is most likely due to ciclosporin partioning into the hydrophobic environment of the membrane. Attempts to identify a membrane protein with ciclosporin-binding capacity using the ciclosporin-affinity matrix were negative. Furthermore, only two erythrocyte membrane proteins, the anion transport protein and glycophorin, are present at levels (approximately $1 \cdot 10^6$ copies of each protein per membrane [15,16]) which could reasonably accomodate the observed ciclosporin binding. The low level of ciclosporin-membrane binding observed before the saturation of the cytosol could be due to the presence of a ciclosporin-binding protein existing at levels below the limits of detectability of the techniques used or it may represent ciclosporin molecules in transit across the membrane.

The existance of a two component uptake of ciclosporin has been previously described in nucleated cells [17,18]. In both studies a higher affinity saturable component and a low-affinity non-saturable component were reported. In addition, as was seen in erythrocytes, the higher affinity component was associated with the cytosolic uptake of the drug. The studies on erythrocytes therefore agree with these previous findings and extend them by ascribing the low-affinity binding to a high-capacity membrane uptake.

At immunosuppressive dosages of ciclosporin, which result in blood concentrations in the range of 0.5 μg/ml [9], the erythrocyte's cytosolic ciclosporin-binding component predominates and is therefore of greater interest. To investigate whether haemoglobin was the ciclosporin-binding moiety within the cytosol as has been suggested previously [9], a competition assay of radiolabelled ciclosporin-binding to erythrocytes was performed. The binding capacity of several protein solutions was determined by this assay. Among two commercial haemoglobin preparations tested only one (Serva) showed ciclosporin-binding capacity, the other haemoglobin (Sigma) was inactive. Whole plasma showed binding capacity as expected [9], as did the human erythrocyte lysate.

Identification of the erythrocyte cytosolic binding protein by affinity chromatography using a ciclosporin-Affigel matrix revealed the existance of a 16 kDa ciclosporin-binding protein. This protein, which could be extracted from the human erythrocyte lysate and–to lesser extent–from one haemoglobin preparation (Serva), had an electrophoretic mobility identical with that of cyclophilin which was previously identified as the major ciclosporin-binding protein of lymphoid cells [3]. This protein is similar in size to the erythrocyte binding protein described by Agarwal et al. [13] who used gel exclusion chromatography for the protein's isolation. Furthermore, by probing with rabbit anti-bovine cyclophilin it was shown that the erythrocyte ciclosporin-binding protein was immunologically cross-reactive with the bovine protein and we therefore conclude that the erythrocyte protein is cyclophilin. The possibility that the erythrocyte ciclosporin-binding protein

was calmodulin, which has recently proposed as a ciclosporin-binding protein [19], is unlikely as the anti-cyclophilin antibody showed no cross-reactivity with calmodulin and ^{125}I-calmodulin showed no binding to the ciclosporin-Affigel (results not shown). The relationship between erythrocyte cyclophilin and the ciclosporin-binding capacity of erythrocyte lysates and commercial haemoglobin preparations was confirmed by the LH column assay (Table II). This showed that the amount of cyclophilin that was extractable from a preparation, using the ciclosporin-Affigel, correlated with the preparations's ciclosporin-binding capacity and absorption of cyclophilin by ciclosporin-Affigel resulted in the total loss of ciclosporin-binding activity of the solutions. The presence of cyclophilin within the erythrocyte was not unexpected as a wide tissue distribution of this protein in nature and occurrence throughout the eukaryotic evolution has been shown [20]. The role of cyclophilin within cell physiology is unknown. Its wide ubiquitous occurrence, high intracellular concentration and evolutionary conservation suggest a critical role in cellular function.

Erythrocytes may represent the major carrier for the distribution of ciclosporin to target sites in the body. Our preliminary studies indicate that ciclosporin diffuses passively into erythrocytes, as the transport is not affected by metabolic inhibitors and only retarded by low temperatures (unpublished observations). Cytosolic accumulation of ciclosporin is due to the presence of the binding protein, cyclophilin. The composition of the extra-cellular space (cyclophilin and possibly other ciclosporin-binding proteins) appears to determine the rate of release of ciclosporin from erythrocytes to the extracellular space (unpublished observations). A shuttle role for erythrocytes in biodistribution of ciclosporin could therefore be envisaged.

High intraerythrocytic ciclosporin concentrations may be also involved in the compound's anti-malarial effects [21,22] which appear to occur on the erythrocytic stages of the parasite [23]. This anti-malarial effect could be due to a direct toxic effect on the parasites, which–by inference from the work of Koletsky et al. [20]–are very likely to contain cyclophilin. Alternatively, the binding of ciclosporin to the erythrocyte cyclophilin may result in some indirect toxic effect on the parasite.

In summary, cyclophilin was shown to be the erythrocyte cytosolic ciclosporin-binding protein with approximately $2 \cdot 10^5$ copies of cyclophilin per cell. The relevance of the existance of such a large pool of ciclosporin-binding protein on immunosuppressive activity, toxicity and biodistribution of the drug needs to be assessed.

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