*Parasite Immunology* 1987, **9**, 737–746

# Topography of epitopes on a polymorphic schizont antigen of *Plasmodium falciparum* determined by the binding of monoclonal antibodies in a two-site radioimmunoassay

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Accepted for publication 24 June 1987

*Summary* The topographic distribution of common and variant epitopes on two divergent allelic forms of the 185-205K schizont glycoprotein of *Plasmodium falciparum* were studied by a two-site radioimmunoassay using monoclonal antibodies. Similarities in the conformation of the two molecules were apparent. On both antigens two distinct regions were mapped, each comprising of both strain-common and polymorphic epitopes. Epitopes common to the two PSAs were found to be closely associated with different variable epitopes in tertiary structure. It is suggested that this may contribute to parasite evasion of the host immune response.

Keywords: *Plasmodium falciparum*, monoclonal antibodies, radioimmunoassay, epitopes, polymorphic schizont antigen

## Introduction

An antigenically variable glycoprotein referred to variously as the p195 (Holder & Freeman 1982, 1984), p190 (Hall *et al.* 1984a) and the polymorphic schizont antigen (PSA) (McBride, Newbold & Anand 1985), is synthesized late in the asexual blood cycle of the human malarial parasite *Plasmodium falciparum*. This molecule is the precursor to several antigens subsequently identifiable on the surface of invasive merozoites (Holder & Freeman 1984, Holder *et al.* 1987, McBride & Heidrich 1987). Immune human sera originating from malaria endemic areas precipitate this antigen (Hall *et al.* 1984a), and human monoclonal antibodies directed against the PSA inhibit merozoite reinvasion *in vitro* (Schmidt-Ullrich *et al.* 1986). When purified PSA is injected into Saimiri monkeys a degree of protection against challenge with live parasites is demonstrable (Hall *et al.* 1984b, Perrin *et al.* 1984). This molecule is therefore considered a potential vaccine constituent.

*P. falciparum* strains have been classified into seven serotypic groups according to their differential reactivity with a panel of mouse anti-PSA monoclonal antibodies (MoAbs) in an immunofluorescence assay (McBride *et al.* 1985). All isolates also have

some PSA epitopes in common, indicating that the molecule has conserved and variable antigenic domains. Recently, comparisons of the amino acid sequences of several PSA variants, deduced from the nucleotide sequences of their genes, have shown the molecule to comprise a number of conserved and semi-conserved regions interspersed with seven variable regions (Weber, Leininger & Lyon 1986, Tanabe *et al.* 1987). The primary structure suggests that more than one of the variable or common peptides may be exposed and accessible to antibody binding on the surface of the native antigen. However, the native conformation of PSA is unknown, and as yet no predictions can be made with regard to the topography of those regions of the molecule that are exposed as antigenic determinants.

Here, a panel of mouse MoAbs specific for a range of strain-common and polymorphic epitopes of PSA was used in a two-site radioimmunoassay (Buranakitjaroen & Newbold 1987) to estimate the number and topographical relationship of antigenic regions on two allelic forms of PSA.

## Materials and methods

### IN-VITRO CULTURE OF *P. FALCIPARUM* AND PREPARATION OF SCHIZONT LYSATE CONTAINING PSA

Cloned *P. falciparum* strains designated T9/94 and T9/96 (Thaithong *et al.* 1984) were used as the source of two serologically distinct, allelic forms (McBride *et al.* 1985, Schwarz *et al.* 1986) of the polymorphic 185-205K schizont glycoprotein, referred to here as the PSA. The parasites were grown in continuous cultures at 5% haematocrits as described by Trager & Jensen (1976). Cultures of 100 ml were synchronized according to Lambros & Vanderberg (1979), and schizonts harvested by density gradient centrifugation on Percoll (Howard & Reese 1984). Percoll harvests, enriched on average to 70% schizont parasitaemias were pelleted, resuspended to 50% v/v in RPMI 1640 and frozen at −70 °C. For preparation of lysates containing PSA antigen schizonts were extracted for 1 h at room temperature with 1% NP-40 (BDH) in 50 mM Tris/HCl pH 8.0 buffer, containing 5 mM EDTA, 5 mM EGTA, 1 mM PMSF and 0.1 mM TLCK and sonicated twice for 5 s at an amplitude of 5 microns with an 8 mm probe. The extract was centrifuged at 10 000 *g* for 5 min and the soluble supernatant diluted in extraction buffer to a final volume 50 times that of the original schizont pellet.

### MONOCLONAL ANTIBODIES

Mouse MoAbs specific for the PSA antigen of *P. falciparum* were raised and characterized as described elsewhere (McBride *et al.* 1985). MoAbs 9.8, 12.4, 12.8, 12.10, 2.2 and 7.5 recognize strain-common epitopes present on more than 300 strains tested to date. MoAb 13.2 is specific for a polymorphic site detectable by IFA on both parasite clones T9/94 and T9/96. MoAbs 7.3, 7.6, 6.1, 13.1 and 17.1 react only with epitopes of the T9/94 clone while the MoAbs 9.2, 12.1 and 12.2 react specifically with the T9/96 clone. In addition to different strain reactivities, in immunofluorescence assays some of the MoAbs also react with different parasite stages. While all the MoAbs react with schizonts and merozoites, only four (MoAbs 2.2, 7.5, 12.8, 12.10) recognized epitopes also present on rings

**Table 1.** Reactivity of anti-PSA monoclonal antibodies

| MoAb | Immunizing strain | Strain distribution* | Stage specificity* | Fragment allocation† |
|---|---|---|---|---|
| 9.8 | PB1 | c (94, 96) | sm | Unknown |
| 12.4 | T9/96 | c (94, 96) | sm | Unknown |
| 7.3, 7.6 | K1 | p (94) | sm | Unknown |
| 9.2 | PB1 | p (96) | sm | Unknown |
| 13.2 | T9/94 | p (94, 96) | sm | 80K |
| 12.1, 12.2 | T9/96 | p (96) | sm | 80K |
| 6.1, 17.1 | K1 | p (94) | sm | 40K |
| 13.1 | T9/94 | p (94) | sm | 40K |
| 2.2, 7.5 | K1 | c (94, 96) | smr | 40K + 16K |
| 12.8, 12.10 | T9/96 | c (94, 96) | smr | 40K + 16K |

* McBride *et al.* (1985); † McBride & Heidrich (1987); Clark, unpublished observations. p, polymorphic epitope; c, strain common epitope; s, schizont; m, merozoite; r, ring; (96, 94), strains on which epitopes are present.

(McBride *et al.* 1985). Furthermore, some epitopes have been mapped by immunoprecipitation and immunoblotting to processed fragments originating from sequentially distant regions of PSA (McBride & Heidrich 1987, Holder *et al.* 1987, Lyon *et al.* 1987). These findings are summarized in Table 1. In this study the epitopes of the PSA will be referred to by the same code numbers as the MoAbs which define them.

### PURIFICATION AND RADIOIODINATION OF MONOCLONAL ANTIBODIES

Immunoglobulins were affinity purified from mouse ascitic fluid on Protein A-Sepharose (Pharmacia), according to Ey, Prowse & Jenkin (1978). The IgG containing fractions were pooled and dialysed against PBS pH 7.3 (Oxoid Dulbecco's A) containing 0·01% azide, and their purity assessed by SDS-PAGE. L and H immunoglobulin chains were the only protein species detectable. Purified immunoglobulins were concentrated by ultrafiltration in 'Centroflo' membrane cones (Amicon) to 1 mg/ml. Antibody activity was determined by immunofluorescence titration against acetone fixed T9/94 and T9/96 cultures. Titres of individual antibody preparations ranged from 1 in 1000 to 1 in 10 000. The antibodies were aliquoted and frozen at −70°C.

Purified immunoglobulins were labelled at room temperature to high specific activity with $^{125}I$ by the Iodo-gen (Pierce) method of Fraker & Speck (1978). One microgram of Iodo-gen (100 $\mu$l of 10 $\mu$g/ml solution in chloroform) was dried on to the surface of a conical bottomed Eppendorf tube under a stream of nitrogen gas, immediately prior to use. Fifty micrograms of purified antibody, 1 mg/ml in PBS and 300 $\mu$Ci of $^{125}I$ (Amersham, IMS-30) were added and the mixture agitated for 10 min. Labelled MoAb was separated from free isotope by gel filtration on a G-25, PD-10 column (Pharmacia), pre-equilibrated with PBS containing 20 mM KI and 0·1% NP-40. Fractions of 0·5 ml were collected and assayed for label incorporation. The specific activity of the labelled antibody ranged from 1 to $5 \times 10^8$ ct/min/$\mu$g protein.

### TWO-SITE RADIOIMMUNOASSAY

The two-site assay was adapted from the procedures outlined by Buranakitjaroen & Newbold (1987). All tests were performed in triplicate. Wells of PVC microtitre plates (Dynatech) were coated overnight at 4 °C with 50 $\mu$l of cold anti-PSA MoAB (50 $\mu$g/ml in PBS). All subsequent steps were performed at room temperature. Plates were washed five times in wash buffer (150 mM NaCl, 50 mM Tris, 0·1% Tween pH 7·5) and blocked with 'Blotto' (5% low-fat dried milk, 0·1% Tween in PBS pH 7·3) for 30 min. Schizont lysate (50 $\mu$l) was added to each antibody coated well and incubated for 60 min, with intermittent mixing, thus binding the PSA to the coating MoAbs. Wells were washed five times in wash buffer, blocked as previously, and probed with 50 $\mu$l of an iodinated antibody, containing $10^6$ ct/min, for 60 min. Finally, plates were washed as before and individual wells excised and counted for 1 min.

## Results

### RATIONALE BEHIND AND PARAMETERS FOR DETERMINING INHIBITION IN THE TWO-SITE ASSAY

It was assumed that the binding of the PSA antigen to the coating MoAb via one epitope would prevent the subsequent binding of labelled antibody probes to the same or proximate epitopes, whilst binding of probes to spatially distant epitopes would be unaffected. Both these assumptions were confirmed by the finding that in all tests the

**Table 2.** Two-site radioimmunoassay on the PSA of clone T9/94

| | Coating antibody | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $^{125}$I-MoAb probe | 7.3 | 7.6 | 6.1 | 13.1 | 17.1 | 13.2 | 12.10 | 2.2 |
| 9.8 | — | — | + + + | + + | + + + | + + | + + + | + + + |
| 12.4 | — | | + + | + | + + + | + + | + + | + |
| 7.3 | — | — | + + + | + + + | + + + | + + + | + + + | + + |
| 7.6 | — | — | + | + | + + + | + | + | + |
| 6.1 | + + + | + + + | — | — | — | + + + | + + + | + + |
| 13.1 | + + + | + + + | — | — | — | + + + | + + + | + + |
| 17.1 | + + + | + + + | — | — | — | + + + | + + + | + + |
| 13.2 | + + | + + + | — | + + | + + + | — | + | + + |
| 12.8 | + + | + + + | — | — | + + + | — | — | - |
| 12.10 | + + | + + | + + | + + | + + + | + | - | + |
| 7.5 | + + + | + + + | + + | + | + + + | + | — | + + |
| 2.2 | + + + | + + + | + + + | + + | + + + | + + + | + + + | — |

The amount of radiolabelled MoAb bound is scored as follows: + + +, ⩾5000 ct/min; + +, 2500–5000 ct/min; +, 1000–2500 ct/min; —, < 1000 ct/min.

**Table 3.** Two-site radioimmunoassay on the PSA of clone T9/96

| $^{125}$I-MoAb probe | Coating antibody | | | | |
|---|---|---|---|---|---|
| | 9.2 | 12.1 | 12.2 | 13.2 | 2.2 |
| 9.8 | — | + | ++ | + | + |
| 12.4 | — | + | + | — | ++ |
| 9.2 | — | + | ++ | + | + |
| 12.1 | ++ | — | +++ | ++ | + |
| 12.2 | ++ | ++ | — | + | + |
| 13.2 | + | ++ | ++ | – | + |
| 7.5 | + | — | — | — | ++ |
| 12.8 | ++ | ++ | ++ | — | — |
| 2.2 | ++ | + | + | + | — |

The amount of radiolabelled MoAb bound is scored as follows: +++, ≥5000 ct/min; ++, 2500–5000 ct/min; +, 1000–2500 ct/min; –, <1000 ct/min.

binding of the radiolabelled probes was inhibited when the coating and radiolabelled MoAbs were identical, while a number of other, non-homologous probes bound to the immobilized antigen well. No evidence was therefore obtained that any of the MoAbs employed in this study were directed against a repeating epitope (Tables 2 & 3).

Background binding of labelled anti-PSA probes was ascertained in control wells coated with MoAb 12.3 and an antigen of 43K, distinct from PSA or any of its processing products (Wilson & Clark, unpublished observations). In these controls the binding of labelled anti-PSA MoAbs never exceeded 500 ct/min. Similarly, when the coating anti-PSA MoAb was homologous with the labelled probe, the binding of the probe did not exceed 500 ct/min/well. Therefore in any assay, activities in excess of 1000 ct/min were considered as positive binding. In the absence of quantitative values for the amount of antigen bound by each coating MoAb no measure of partial inhibition of the binding of labelled probes was attempted. Deductions concerning the spatial relationships between epitopes were made only where the binding of the labelled probes was reduced to less than twice background, i.e. maximum inhibition was occurring.

Since the antigen for the present experiments was derived from early schizonts, it was assumed to consist of the intact precursor rather than to contain its processed fragments. This was strongly supported by the general finding that PSA immobilized by epitopes known to be located on one or another of three different fragments (Table 1), still contained marker epitopes of the other fragments and did bind most of the corresponding antibody probes (Tables 2 & 3).

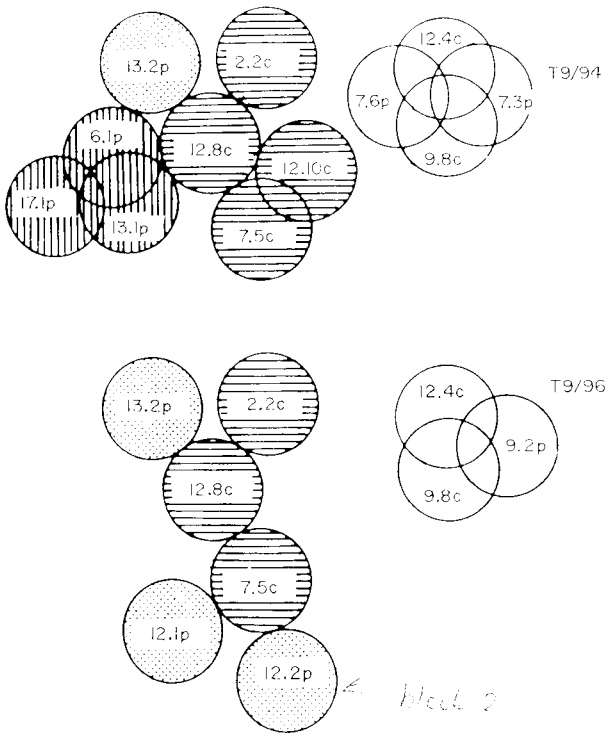


**Figure 1.** Topography of epitopes on the polymorphic schizont antigens of *P. falciparum* clones T9/94 and T9/96. Individual epitopes are represented by circles: c, common; p, polymorphic. Overlapping circles represent epitopes which are indistinguishable in the two-site assay, while circles which touch inhibit the binding of monoclonal antibody to one another. Circles not in contact represent spatially distant epitopes as ascertained by the two-site assay. Allocation of individual epitopes to PSA fragments, following processing of the molecule, is indicated by shading. Fragments: ▨, 80K; ▩, 40K; ▧, 16K; □, unknown.

### BINDING OF RADIOLABELLED PROBES TO MONOCLONAL ANTIBODY-IMMOBILIZED PSA OF CLONE T9/94

Clone T9/94-derived PSA was immobilized via the epitopes 7.3, 7.6, 6.1, 13.1, 17.1, 13.2, 12.10 or 2.2, and then probed separately with all of the labelled anti-PSA MoAbs in order to discern the spatial relationship between the epitopes. The results are presented in Table 2, and diagrammatically in Figure 1. The epitopes appear to segregate into three spatially distinct clusters.

The first is comprised of conserved sites 9.8 and 12.4 and the polymorphic determinants 7.6 and 7.3. Reciprocal blocking of the 7.3 and 7.6 epitopes by the respective antibodies and also lack of binding of 12.4 and 9.8 MoAb probes to 7.3- or 7.6-immobilized PSA, indicates that these four antibodies define closely situated epitopes.

The second cluster comprises of polymorphic epitopes 6.1, 13.1 and 17.1. These three MoAbs inhibit the binding of each other and may define a single epitope or epitopes located in close proximity. Since blocking of the first group of epitopes has no effect on the

binding of MoAbs 6.1, 13.1 or 17.1 and *vice versa*, the respective groups are non-identical and spatially distant.

The third cluster contains the common epitopes 12.8, 12.10, 7.5 and 2.2, known to be located at the C-terminus of PSA and, unexpectedly, the polymorphic site 13.2, known to be in the N-terminal region. Central to this cluster appears to be the 12.8 epitope, as binding of the 12.8 probe is abrogated when the 13.2 , 12.10 and 2.2 sites are occupied by antibody. However, blocking of the 2.2 and 12.10 epitopes has little effect on the binding of 13.2 MoAb and *vice versa*, indicating that they occupy more distant, non-overlapping sites around the 12.8 epitope. The epitopes 7.5 and 12.10 appear to be located closer to each other and to 12.8 than to the 2.2 epitope. This third cluster of epitopes appears to overlap partially the second cluster (6.1, 13.1). This is indicated by inhibition of the binding of the 12.8 probe when the 6.1 and 13.1 sites are occupied, and also by binding of the 13.2 probe which is abrogated when the 6.1 site is blocked.

### BINDING OF RADIOLABELLED PROBES TO MONOCLONAL ANTIBODY-IMMOBILIZED PSA OF CLONE T9/96

The PSA variants of *P. falciparum* clones T9/94 and T9/96 share epitopes 9.8, 12.4, 13.2, 2.2, 7.5 and 12.8. To determine whether the topology of these epitopes could be confirmed on another PSA variant, two-site assays were performed with PSA from clone T9/96. In addition, MoAbs 9.2, 12.1 and 12.2 which define polymorphic epitopes specific to the T9/96 variant were also included. Results are presented in Table 3 and Figure 1.

General similarities are observed in the spatial distribution of epitopes on the two variant molecules. As for T9/94 PSA, the epitopes on T9/96 PSA segregate into two independent, non-overlapping clusters containing the strain-common epitopes. The first cluster is comprised of strain common epitopes 12.4 and 9.8, and includes also a polymorphic epitope 9.2. The second cluster again encompasses the strain-common epitopes 2.2, 7.5 and 12.8 located at the C-terminus and the polymorphic 13.2 epitope of the N-terminal region. Two additional polymorphic epitopes 12.2 and 12.1, which have also been mapped to the 80K N-terminal region, do not appear analogous in position to any of the epitopes mapped on the T9/94 molecule (Table 2). However, since blocking of either of the epitopes inhibits the binding of the 7.5 probe, they are included in the same cluster here. The 12.1 and 12.2 epitopes appear distant from each other and also from the 13.2 site.

## Discussion

The two-site RIA, whereby MoAbs were used to probe the spatial relationship between epitopes on the polymorphic schizont antigen of *P. falciparum*, provided some novel findings on the topography of the molecule. An interpretation of the results for two variants of the antigen is presented as a model epitope map in Figure 1.

The present results may be considered in the light of evidence for the structural and serological polymorphism of the antigen. The PSA gene from four strains of *P. falciparum* has been sequenced, revealing that it exists in two radically different allelic forms, between which there is evidence for limited recombination (Holder *et al.* 1985, Mackay *et al.* 1985, Weber *et al.* 1986, Tanabe *et al.* 1987). Comparisons of the deduced amino acid structure

of the gene products have shown that the two extreme forms share five conserved regions which are interspersed by numerous blocks of variable sequences. The two major versions of the gene products are easily distinguished, and each can be further subtyped serologically with MoAbs specific for polymorphic epitopes (McBride, Walliker & Morgan 1982, McBride *et al.* 1985). The PSAs of strains T9/94 and T9/96 were used as representative examples of the two contrasting allelic products (McBride *et al.* 1985, Schwarz *et al.* 1986, Tanabe *et al.* 1987).

In spite of the well-documented non-identity of the two PSA variants, the present study indicates that at least two features of their topography are very similar. First, on both antigens we find two non-overlapping regions containing conserved epitopes. These regions are separated physically following cleavage of the PSA into several antigens on the merozoite surface. One of the regions (defined by epitopes 9.8 and 12.4) is lost at the time of erythrocyte invasion, while the other (defined by epitopes 7.5, 12.8, 12.10 and 2.2) remains throughout invasion on a 16–19K fragment cleaved from the C-terminus of PSA (McBride & Heidrich 1987, Holder *et al.* 1987, Clark & McBride, unpublished observations). This fragment contains a large block of conserved sequence, including several cysteine residues (Tanabe *et al.* 1987) which appear to stabilize the four conserved epitopes studied here (McBride & Heidrich 1987). As indicated in Figure 1 these four epitopes are adjacent but non-identical (e.g. epitope 2.2 is distinguishable from epitopes 12.8, 12.10 and 7.5). The relative positions of these epitopes appear to be the same for both the PSA variants studied, thus further extending the evidence for the conserved organization of this region of the molecule.

Secondly, within both of the topographical areas mapped, certain conserved epitopes were found in proximity to polymorphic determinants. Thus the conserved sites 9.8 and 12.4 appear to occupy a position close to the polymorphic epitopes 9.2 (on T9/96) or 7.3 and 7.6 (on T9/94). Similarly, at the C-terminus the conserved sites 12.8 and 7.5 appear to be located proximal to one or more different polymorphic epitopes. Such proximity could arise either from the epitopes being formed by adjacent continuous amino acid sequences or from sequences distant in primary structure but closely associated in tertiary structure. It is more likely the second mechanism which contributes to the observed clustering of the polymorphic sites around the conserved epitopes at the C-terminus. For example, although the sequential polymorphic epitopes 6.1 and 13.1 map to a variable segment preceding the conserved part of the C-terminus, they are eventually cleaved from the 12.8 site (McBride & Heidrich 1987) and therefore probably not directly linked in primary sequence (Tanabe *et al.* 1987).

Other polymorphic sites, 13.2 (on T9/96 and T9/94) and 12.1 and 12.2 (on T9/96) map to the 80K peptide (McBride & Heidrich 1987, McBride & Clark, unpublished observations) from the N-terminal region of PSA (Holder *et al.* 1985, 1987), and their apparent proximity to sites 12.8 and 7.5 respectively, from the C-terminal region can be explained only as resulting from tertiary conformation.

There are two limitations inherent in the present approach. First, PSA is a membrane molecule, and detergent extraction with NP-40 could alter its conformation. Second, processing of PSA into its product antigens may lead to subtle changes in the organization of epitopes on merozoites. As far as discernible by reactivities with the specific MoAbs, conformations of all epitopes studied here on the extracted precursor were comparable to those detectable on the precursor or its fragments *in situ* on fresh unfixed schizonts or merozoites respectively, by immunofluoresence (McBride *et al.* 1985, McBride &

Heidrich 1987). In the absence of any contrary evidence it is therefore assumed that the present model of PSA topography may be essentially valid for the native antigen.

It is tempting to speculate on the significance of the present observations in terms of immunity to PSA. Should the polymorphic epitopes in the vicinity of the conserved sites be immunodominant, binding of antibodies to the former could interfere with recognition of the latter. Such interference could be mediated by antibody binding to a highly variable peptide such as the N-terminal 80K fragment (Lyon *et al.* 1986, Weber *et al.* 1986, Tanabe *et al.* 1987) that, in addition, is shed from merozoites at the time of reinvasion (Holder *et al.* 1985, Lyon *et al.* 1987) and does not apparently contribute to the antibody-mediated blockade of erythrocyte invasion *in vitro* (Strych *et al.* 1987). This may be useful in blocking antibody responses to conserved peptides, which may have a biological function, for example, the 16K glycoprotein, which remains on the surface of merozoites throughout invasion.

This hypothesis is now being tested by determining which MoAbs against strain-common epitopes inhibit merozoite invasion *in vitro*, and whether this can be prevented in the presence of MoAbs directed against the polymorphic sites.

## Acknowledgements

This investigation was supported by the Wellcome Trust and by the UNDP/World Bank/WHO Special Programme for Reseach and Training in Tropical Diseases, under the Scientific Working Group on the Immunology of Malaria. We thank David Walliker for parasite clones T9/96 and T9/94, Chris Newbold and Peira Buranakitjaroen for help and advice with the two-site assay, and Edinburgh and S.E. Scotland Blood Transfusion Service for the provision of human blood and serum.

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