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NATIONAL BIOLOGICAL STANDARDS BOARD

Scientific Policy Advisory Committee

REVIEW OF THE DIVISION OF HAEMATOLOGY

A number of exhibits illustrating the work of the Division will be available for view at NIBSC on 9 December

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INTRODUCTION

1. OVERVIEW OF THE DIVISION'S FUNCTIONS

The overall functions of the Division have remained broadly unchanged since the last SPAC Review in 1990, and consists essentially of control and standardisation activities and relevant research relating to biological drugs used to treat haematological disorders. The majority of these drugs fall into the scientific area of haemostasis and thrombosis, and this has continued to be the main theme of the Division's work. However, since the last review, the links between NIBSC and the UK Blood Transfusion Service have been strengthened and some additional work has been initiated in the Division in the area of Transfusion Medicine.

In terms of numbers of batches of products examined, the Division's control work is the most extensive of any the Institute's departments. Since the last Review, the overall control workload has remained fairly constant at approximately 650 batches/year; a fall in the number of batches of Factor VIII (FVIII) submitted during the latter part of the review period has been balanced by an increase in the number of batches of low molecular weight (LMW) heparin. The Division continues to meet a high and increasing demand for its standards, paricularly for clotting factors; over 30% of all standards issued from the Institute originate from the Division, and International and British Standards for clotting factors are the most frequently requested of all the Institute's standards.

Despite this heavy demand for control and standardisation work, the Division has historically been one of the smallest of the major scientific Divisions in terms of accommodation and staff. This imbalance has to a certain extent been redressed since the move to South Mimms in 1987 by the appointment of more scientific staff and the acquisition of extra laboratory space, but the accommodation and staff needs for the future remain a concern and are addressed towards the end of this report.

The previous Head of Division, Dr Thomas, retired in September 1990, and Dr T W Barrowcliffe, previously a senior scientist in the Division, was appointed in his place. Dr Barrowcliffe took up his appointment in January 1991, and following a thorough review of the Division's activities made some changes in the way the work is organised. This involved mainly the establishment of 4 broad programme areas under which all the work is categorised.

The work is now organised under the following 4 headings:-

1) **Blood Products**

This encompasses 2 types of product, albumin solutions and clotting factor concentrates, which together account for the bulk of control work in the Division. Clotting factor concentrates are used to treat congenital haemorrhagic diseases, primarily haemophilia A and B, and this field has undergone rapid development during the review period, with the advent of new high-purity products from plasma, and the succesful introduction of recombinant FVIII.

Clotting factors are priced by the unit so that incorrect potency assignments have financial as well as clinical consequences; it is estimated that since 1980 over £1 000 000 has been saved from the Department of Health's bill for FVIII as a result of the Division's control activities. Albumins are given in large volume for plasma exchange or volume replacement in trauma patients, and the detection of minor contaminants continues to be an important safety aspect.

Viral safety is a major concern with all blood products and an important development which had just been initiated at the last review has been the testing of plasma pools from which products are derived for viral markers. The testing is carried out in the Divisions of Virology and AIDS, but the Division of Haematology is responsible for co-ordinating all the results for the products it controls before release and decisions on the release of each batch are made by the Head of Division.

2) **Antithrombotic drugs**

Thrombosis and its sequelae continue to be the major cause of morbidity and mortality in the UK, and drugs used to treat or prevent this condition play a very important role in public health. Heparin is the oldest established antithrombotic agent and, although it was first used clinically in the 1930's, research into its mechanism of action is still continuing. As anticipated in the last Review, LMW heparin has become an important new adjunct to the traditional "unfractionated" heparin, and our control and pre-licensing work in this area has substantially increased.

A new range of potential antithrombotic drugs, many prepared by recombinant DNA technology, is under development, and of these hirudin is the the most advanced, with others such as protein C and tissue factor pathway inhibitor (TFPI) also under intense investigation by several companies. An increasing proportion of the Division's work in this area is concerned with research and development of appropriate methodology relating to these new agents.

3) **Thrombolytic Drugs**

These clot-dissolving agents are potential life-saving drugs if given under appropriate conditions in acute myocardial infarction, which accounts for almost 100 000 deaths annually. The newest of these drugs, tissue plasminogen activator (tPA), which was the first thrombolytic agent to be manufactured by recombinant DNA technology, has not fulfilled its expected therapeutic potential, and there is intense commercial and clinical interest in modified forms of tPA and other agents such as single-chain urokinase-type plasminogen activator (SCuPA). The mechanisms by which these drugs act are quite complex and not fully established, and therefore it is important for the Division to maintain a research-based strategy in this area so that appropriate control methodology can be developed.

4) Transfusion Medicine

Following the collaboration which was origanally set up between NIBSC and the UK Blood Transfusion Service (BTS) with the main aim of establishing UK Guidelines, work on transfusion medicine in the Institute has increased. At present there is no separate department of transfusion medicine and the work in this area is carried out in 3 different Divisions. The Division of Virology is responsible for standardisation of tests for viral markers used in donor screening; the Standards Division is responsible for operating the NEQAS and preparing reference reagents in the field of blood group serology; the Haematology Division is responsible for research and development in relation to therapeutic products produced by the BTS. The work on virology has recently been reviewed by SPAC during its review of that Division and will not be considered here. The work on blood group serology will be considered in the present review and is described in the report under the Transfusion Medicine Section (Section 4).

Since the last review, the work in the Haematology Division on anti-D has been extended, and following discussions with the Transfusion Service, some new work has been initiated on platelets. In order to establish the platelet programme, Dr W Ouwehand, a Consultant Haematologist at the Cambridge Regional Tranfusion Centre and lecturer in the Department of Transfusion Medicine at the University of Cambridge, was appointed to the Division on a part-time basis. A scientist has just been appointed to help develop the work in this area.

The aim in setting up these broad programme areas was 2-fold: to encourage collaboration between scientists within the Division and foster a sense of teamwork, and to streamline and simplify financial control, especially in relation to new financial arrangements for the Institute with several Service Level Agreements for separate areas of work. The Head of Division considers it important for scientists to look beyond their own areas of expertise and interests and take advantage of other techniques and areas of knowledge in order to build up a more complete biological picture of the substances they are working with. For example, the Division's work on recombinant hirudin, an antithrombotic drug, has covered its antithrombotic action *in vivo*, overall anticoagulant activity in plasma, enzyme kinetics of its interaction with thrombin, and standardisation of potency measurement, and this has involved 3 scientists, Dr Gray, Dr Longstaff and Dr Gaffney, pooling their resources. Collaboration with scientists in other Divisions is also encouraged, and there are several such collaborative projects, which are described in detail in the report.

2. STAFF

Name	Grade
Scientific Staff	
Dr Trevor W Barrowcliffe	Special Appointment (Head of Division)
Dr Patrick J Gaffney	Special Appointment
Dr Anthony R Hubbard	Grade I Scientist
Dr Elaine Gray	Grade I Scientist
Dr Geoffrey Kemball-Cook	Grade I Scientist
Dr Colin Longstaff	Grade I Scientist
Dr Susan J Thorpe	Grade I Scientist
Dr Paul Metcalfe	Grade I Scientist
Dr Willem H Ouwehand	
Support Staff	
Mr John B Pring	Higher Scientific Officer (Laboratory Manager and Quality Co-ordinator)
Miss R Elizabeth Merton	Higher Scientific Officer
Mrs Lesley J Kempsford	Higher Scientific Officer
Ms Jill E Tubbs	Higher Scientific Officer
Ms Jane Watton	Higher Scientific Officer
Mrs Lynne J Weller	Scientific Officer
Miss Sally A Bevan	Scientific Officer
Mr Stephen Thomas	Scientific Officer
Miss Tracey A Edgell	Scientific Officer
Administrative Staff	
Mrs Susanne M Barsby	Personal Secretary

Vacancies

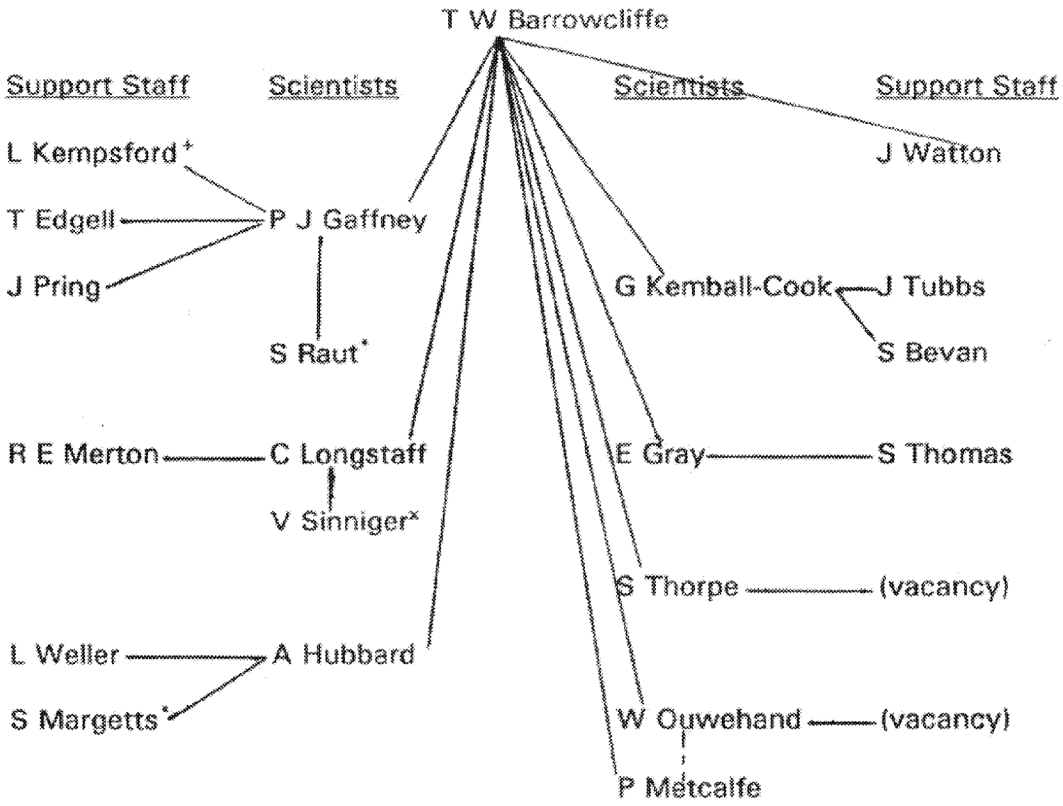
There are 2 current vacancies at Scientific Officer level, one since March 1993 and the other since October 1993.

Externally Funded Staff

Scientific
Dr Sanjeev Raut, Grade I, 3 year appointment from June 1993.
Support
Miss Sarah Margetts, Scientific Officer, 3 year appointment from June 1993.

Visiting Workers
Miss Ana Padilla, Madrid, October 1990 - September 1992
Dr Valerie Sinniger, Paris, March 1993 - August 1994

Organisational Chart



+ Currently on maternity leave * Externally funded
× Visiting worker

Distribution of Responsibilities in Programme Areas

BLOOD PRODUCTS

Scientists: G Kemball-Cook, T W Barrowcliffe, A R Hubbard (part), some support from E Gray

Support staff: J E Tubbs, S Bevan, J Watton (part)

ANTITHROMBOTIC DRUGS

Scientists: E Gray, T W Barrowcliffe, A R Hubbard (part), C Longstaff (part), some support from P J Gaffney

Support staff: S Thomas, J Watton (part), L Weller (part), S Margetts (externally funded)

THROMBOLYTIC DRUGS

Scientists: P J Gaffney, C Longstaff, S Raut (externally funded)

Support staff: L Kempsford, T Edgell, R E Merton, J Pring

TRANSFUSION MEDICINE AND GENERAL HAEMATOLOGY

Scientists: S J Thorpe, P Metcalfe, W H Ouwehand (part-time)

Support staff: None (2 vacancies)

3. ACCOMMODATION

The 20 scientific and support staff (including 2 vacancies but excluding grant supported and visiting workers) are housed in 6 working laboratories comprising approximately 8 units of laboratory space. In addition, there is an equipment room, a cell culture laboratory, a 4°C cold room and another small room shared with the Division of Endocrinology.

Since the last review, one of the 6 working laboratories was created from a plant room but this is less than ½ unit, and offers only approximately 4½ metres bench space. Taking into account 2 additional staff on grant-supported work and one long-term visiting worker, the Division's current accommodation is extremely cramped and there is no space for expansion. This is discussed further at the end of the report.

The Division of Haematology is located in the South Block of the Institute in rooms, 5041, 5042, 5045, 5047, 5050, 5052, 5053, 5054, 5055, 5056, 5059, 5060. The specific locations where various members of the Divison are based, and the general nature of the work carried out is as follows:-

Room 5041 - shared with the Endocrinology Division. Equipment storage and occasional laboratory use by Dr Gaffney's staff

Room 5042 - Fume Cupboard + gel dryer used communally by the Division

Room 5045 - Fibrinolysis - Dr Patrick J Gaffney, Mrs Lesley Kempsford, Miss Tracey Edgell, Dr Sanjeev Raut (externally funded)

Room 5047 - Immunohaematology - Dr Susan Thorpe, Scientific Officer vacancy

Room 5050 - Blood Products - clotting factor concentrates - Dr Geoffrey Kemball-Cook, Ms Jane Watton, Ms Jill Tubbs, Miss Sally Bevan

Room 5053 - Office - Mrs Susanne Barsby

Room 5055 - Head of Division - Dr Trevor W Barrowcliffe

Corridor Office - Mr John Pring (Laboratory Manager)

Room 5056 - Blood Products - albumin, FVII, fibrinolysis - Dr Anthony R Hubbard, Mrs Lynne Weller, Dr Colin Longstaff, Ms R E Merton, Miss S Margetts (externally funded)

Room 5052 - Equipment - Fast Protein Liquid Chromatography, Gamma Counting and centrifuges

Room 5054 - Transfusion Medicine - Dr Paul Metcalfe, Dr Willem Ouwehand (part-time)

Room 5059 - Cell Culture, Polymerase Chain Reaction (PCR)

Room 5060 - Antithrombotic drugs - Dr Elaine Gray, Mr Stephen Thomas, SO vacancy, desk for visiting worker

4. RECOMMENDATIONS OF PREVIOUS REVIEW

(a) that the implementation of recommendations made at the last review in 1985, together with overall management of the Division, had been excellent and that Dr Thomas was to be congratulated;

(b) that haematology was a significantly more important discipline than it had been at the time of the last review, with the Division handling a wide range of new products, including those derived from recombinant technology, and providing valuable advice on licensing and control matters to pharmaceutical companies, the MCA and other organisations;

(c) that the Division's contribution to public health through its collaboration with the UK Blood Transfusion Service was no longer limited to standardisation and control of licensed medicines;

(d) that the balance between research and development and standardisation and control appeared to be satisfactory, given that a clear distinction was difficult to make, and should not be changed at present;

(e) that, although the allocation and modification of priorities were determined mainly by external factors, overall future plans provided a firm base for progress and scientific development within the constraints of statutory obligations;

(f) External Funding

that important research owed much to external funding and this means of securing resources should be encouraged, provided that exploitation of commercial potential was approached through existing mechanisms and only with the Director's approval;

(g) Molecular Biology

that adequate molecular biology facilities were currently available within the Institute for all practical purposes, but additional expertise should be sought, both internally and through external collaboration, and Haematology staff encouraged to develop their skills;

(h) Links with Blood Transfusion Service (BTS)

that active support should be given to strengthening existing collaboration with the BTS in every possible way;

(j) Staffing

that the scientific staff numbers appeared to be appropriate, but may be affected in the medium-term future in the light of (h); (support staff were the subject of a separate review)

(k) Accommodation

that laboratory space was currently adequate but provided little room for growth;

(l) Equipment

that equipment was currently adequate with the exception that an automated coagulometer (ACL) as identified at paragraph 13.4 of the report (cost approximately £24 000) could be acquired when resources permitted.

5. WORK DISCONTINUED OR COMPLETED SINCE PREVIOUS REVIEW

Several areas of work have been discontinued in order to accommodate new projects, and some projects have come to a natural completion, a brief summary of these is as follows:

In **Blood Products**, the work on standardisation of Factor VII (FVII) concentrates has been completed, and the studies on FVII and lipids discontinued. Control work on albumin has been reduced in frequency, and in the FVIII area control work on some

of the older products has also been reduced in order to expand studies of the newer products such as recombinant FVIII.

In **Antithrombotic Drugs**, the basic ultrastructural studies on endothelium carried out by Dr Thomas have been discontinued. Work on dermatan sulphate has been suspended pending the outcome of further clinical studies, and work on the interaction of heparin with lipase enzymes discontinued.

In the **Thrombolytic Drugs** area, no major projects have been discontinued but several ongoing projects have been completed. Work on the carbohydrate analysis of tPA has been suspended because of diversion of resources to other aspects of tPA.

In the **Transfusion Medicine** area, work on the characterisation of the D antigen has been suspended because of difficulties in producing the monoclonal antibodies required, and the project on blood group antigens in cardiac muscle has been completed. In General Haematology the vitamin B12 standardisation work has been completed.

6. NEW WORK STARTED SINCE THE LAST REVIEW

Although most of the work of the Division was ongoing at the time of the last review, some new areas have been opened up in response to changing needs, mostly connected with the development of new products. Several of these areas involve the use of molecular biology techniques as recommended in the last review. The projects are described in detail in the report, but a brief summary is given here.

In **Blood Products**, a project on the molecular biology of FVII was carried out with the co-operation of the Haemostasis Research Group, Northwick Park. A project was initiated on the preparation of human antibodies to FVIII using the molecular genetic approach developed by Dr Winter's group in Cambridge. Studies of the thrombogenicity of high-purity Factor IX (FIX) concentrates and on Factor XI (FXI) concentrates, both new products, were set up. A project on the characterisation of antithrombin III (AT III) concentrates by monoclonal antibodies was initiated.

In **Antithrombotic Drugs**, new standardisation work on LMW heparin was carried out in response to a request from the European Pharmacopoeia (EP), and work on a proposed new unit for heparin has commenced. Standardisation work was initiated on protein C concentrates, protein S in plasma, and TFPI. New collaborative projects were established on the interaction of cytokines and the coagulation system in response to endotoxin, and on interaction of heparin and similar compounds with growth factors.

In **Thrombolytic Drugs**, new enzymolgy and standardisation work has been carried out on SCuPA, and 2 molecular biology-based projects have been initiated on tPA. Standardisation studies have been carried out on a number of genetically modified tPA preparations currently under development as pharmaceutical agents.

In **Transfusion Medicine**, a new anti-D assay technique has been developed and, as already mentioned, a programme of work on platelets established, with the initial emphasis on platelet immunology.

SUMMARY

The work of the Division since the last Review, together with its future scientific priorities, is presented in detail for each of the 4 main programme areas in the following sections. A brief summary highlighting the main achievements of the Division, and indicating the future challenges, is given here.

Control work has been performed on blood products, antithrombotic drugs and thrombolytic drugs, the total number of batches being over 2,400. Problems have been found with potency of some FVIII concentrates, endotoxin contamination in albumins, thrombogenicity in FIX and FXI concentrates, quality of AT III concentrates, and potency of some batches of heparin and LMW heparin.

The Division is responsible for 40 different standards and reference materials, and over 55 000 ampoules have been despatched since the last review. New International Standards have been established for FVIII/von Willebrand factor in plasma, AT III concentrate, Factor VIIa (FVIIa) concentrate, FIX concentrate, α-thrombin, plasma fibrinogen, haemoglobin A_2 and F, and ferritin. Standardisation of recombinant hirudin has been achieved via the use of the α-thrombin standard and a standardised assay method. Problems with the assay of high-purity and recombinant FVIII have been solved with the introduction of internationally agreed assay methodology. Work has been initiated on the standardisation of protein C concentrates, protein S in plasma, TFPI, variant forms of tPA and SCuPA.

New British Standards have been established for FVIII concentrate, Factors II-IX-X concentrate, FVIII plasma, and blood coagulation factors plasma; the latter was calibrated for protein S for the first time. Work has been carried out on behalf of the EP to develop working standards for FVIII and LMW heparin. A new project has been initiated under the European Community's Measurement and Testing Programme, to develop several European reference plasmas for diagnostic use in coagulation testing.

Comparative studies on recombinant and plasma derived FVIII have focused on their polypeptide structure and biological activities. Studies of activated FVIII in concentrates have been carried out *in vitro* and *in vivo*, and the *in vitro* method developed was found to be relevant to the immunogenicity of one product in patients. In collaboration with the Division of Immunobiology, a contaminant in FVIII concentrates which may be responsible for their immunosuppressive actions *in vivo* was identified. A new assay for Factor IXa (FIXa) in concentrates was developed and this was shown to correlate with thrombogenicity *in vivo*; similar studies were also carried out on FXI concentrates. A method was developed to quantitate heparin binding to AT III without the need for purification, and this was applied to characterisation of AT III in concentrates and in plasmas from patients with genetic mutations.

Standardised methods of assay of LMW heparin were developed for the EP, and more physiological methods of assessment of its overall anticoagulant actions, in the presence of platelets and endothelial cells, were explored, in order to establish assays which may correlate better with *in vivo* activity. Studies were initiated on the

mechanism of endotoxin - induced intravascular coagulation, and its possible prevention by antithrombotic drugs.

Studies of the enzymology of plasminogen activators, plasmin, and their inhibitors have been extended, and the role of template mechanisms and conformational changes in the promotional effects of fibrin and heparin on plasminogen activation defined. These studies are relevant to the action of plasminogen activators *in vivo*, and have facilitated the design of more physiologically relevant assay systems for tPA and SCuPA. The inhibitory activity of α_1-antiplasmin has been compared to that of aprotinin and its mechanism of action more clearly defined.

A new assay method for therapeutic anti-D preparations has been developed which has some advantages over the current haemagglutination techniques. A large number of monoclonal anti-blood group antibodies have been screened for reactivity with human tissues and soluble antigens, and the most common cross-reactivities identified. A reference panel of platelets with known specificity for human alloantibodies has been set up, and several workshop exercises organised to improve the detection of clinically important platelet antibodies.

In the future it is anticipated that a number of new therapeutic agents will be developed by recombinant DNA techniques, especially in the areas of antithrombotic and thrombolytic therapy. Some of these will consist of variant molecules which may differ in their biological properties from the natural form, and will require detailed studies in order to develop the most appropriate control and standardisation techniques. The Division will need to develop a flexible research and development strategy to meet these new challenges, whilst maintaining its well established programme to provide standards and control testing for existing materials.

1. **BLOOD PRODUCTS**

INTRODUCTION

The control and standardisation of blood products has remained one of the largest areas of work in the Division. In terms of number of batches blood products account for the bulk of the Division's control work - over 500 batches/year out of the total of approximately 650. The preparation and calibration of British Standards (BS), which require frequent replacement, as well as International Standards (IS), is also a demanding area.

There are 2 major groups of blood products controlled by the Division; clotting factor concentrates and albumin - immunoglobulins are controlled by the Division of Immunobiology. A major concern with all blood products, particularly clotting factor concentrates, is viral safety. Because of the difficulty in applying viral marker tests to the final products, the emphasis of the Institute's programme has been testing of plasma pools. Testing of plasma pools from the UK manufacturers commenced in 1987 and from 1989 samples of pools for each batch of all products were also requested from commercial companies. This work is carried out in the Virology and Aids Divisions, and has been reviewed extensively by SPAC. However, a brief summary of the findings is presented in Table 1. It can be seen that a small number of pools have been found to be positive for each viral marker, despite the fact that they comprise only donations which have been supposedly tested and found negative for these markers. Where positive plasma pools have been found, advice has usually been given to the Medicines Control Agency (MCA) not to release the batch. Recent publicity about the apparent use of non-tested German plasma for manufacture of blood products has highlighted the importance of testing plasma pools as a check on quality control of donor testing.

Table 1 NIBSC testing of plasma pools for viral markers

Viral Marker	Date Testing Started	Number of Pools Tested	Number Found Positive
Anti HIV	1986	6800	2
HBsAg	1986	6800	9
Anti HCV	1993	1350	7

The other main aspects which are important for control are potency (clotting factor concentrates), clinically undesirable contaminants (albumin, FIX and FXI concentrates), and immunological reactions (FVIII concentrates). The Division's strategy is to develop methods which can be used to assess and characterise these aspects and to carry out research which throws light on the mechanisms of the biological activities of these products.

1.1 CLOTTING FACTOR CONCENTRATES

The following products, in order of decreasing frequency of use, are the subject of control and standardisation work in the Division: Factor VIII, Factor IX, Factor VII and VIIa, Antithrombin III, Factor XI and Factor XIII. The largest proportion of the work is on Factor VIII.

1.1.1 Factor VIII Concentrates

1.1.1 (a) Characteristics of Products

Factor VIII concentrates derived from human plasma are used in both prophylactic and intensive replacement therapy of haemophiliacs in the UK. This is a multi-million pound market (over £30 000 000/year) which is served by both NHS-funded plasma fractionators (Bio Products Laboratory (BPL), Elstree; Protein Fractionation Centre (PFC), Edinburgh) and non-UK commercial sources. In addition, a single UK commercial source supplies porcine FVIII concentrates intended for therapy of haemophiliacs who have developed antibodies to human FVIII.

During the period under review, there have been several major changes in the pattern of products submitted to NIBSC for testing (see Table 2).

Table 2 FVIII products submitted to NIBSC for batch testing

	Year			
	1990	1991	1992	1993 (projected)
Human FVIII, NHS (No of batches)	194	188	121	76
Human FVIII, Commercial (No of batches)	30	31	33	54
Commercial batches (% of total)	13%	14%	21%	42%
Porcine FVIII (No of batches)	21	31	36	24
Total No of batches submitted	245	250	190	150
High-purity batches (% of total)	7.8%	9.2%	34%	62%
S-D treated batches (% of total)	0%	7.2%	28%	45%

(i) The total number of batches submitted per year has decreased from about 250 to 150 as a result of larger batch sizes prepared by NHS manufacturers.

(ii) The proportion of 'high-purity' (HP) batches prepared by either monoclonal antibody techniques or 'conventional' chromatography has increased markedly from less than 10% in 1990 to over 60% in 1993; this reflects the growing preference by clinicians for purer products in the belief, not yet proven, that they have less effects on the immune system than the well-established 'intermediate-purity' products (see Section 1.1.1.(e)).

(iii) Solvent-detergent (S-D) viral inactivation technology has been extensively introduced by manufacturers, and 1994 should see the proportion of S-D batches submitted rise to well over 50%.

Recombinant FVIII has been under development for many years, and the first clinical trials were reported in 1989. During the latter part of the review period 2 products were licensed by the FDA in the USA and in Sweden; most recently licence applications for the same products in Europe have been submitted through the CPMP concertation procedure in Brussels. The Division's work on recombinant FVIII commenced in 1989 and has consisted of standardisation and assay methodology, structural studies, immunological aspects and studies of FVIII activation. In all these studies recombinant FVIII has been compared with plasma-derived materials, since a major concern is the extent to which the recombinant and native molecules differ. The studies on recombinant FVIII will therefore be described under these sections.

During the last 18 months discussions have been held with 2 other companies who are developing recombinant deletion mutants of FVIII, but as no samples have yet been released by the manufacturers it has not been possible to start work on these materials.

1.1.1 (b) Batch Control

The Division continues to monitor closely the potency of all products submitted, and to maintain the ability to perform all 3 currently used assay methods. The purchase of an ACL coagulometer, as recommended in the last SPAC review, has greatly helped the routine assay programme, and has improved the precision of the assays.

However the fact that virtually all the Division's assays could be performed on the ACL, and the large amount of work required on heparins, meant that the instrument was constantly in demand. During the last year we were fortunate to acquire a second instrument which is dedicated to the clotting factor work.

The two-stage method (EP reference method) which has continued to be our

The two-stage method (EP reference method) which has continued to be our routine method, was transferred to the ACL after a suitable period of comparative validation against the traditional manual technique. The one-stage method, used by most clinicians and several manufacturers, is also automated on the ACL, and has been used in comparison with the two-stage method for some products. For both these methods we use largely in-house reagents, resulting in considerable financial savings to the Division. Indeed we would not be able to perform all our control and standardisation work within budget if we used solely commercial reagents; the commercial cost of a typical year's assays for FVIII alone is approximately £25 000, whereas the actual cost is less than £5,000.

During the review period, a third type of assay, the chromogenic method (conceptually based on the two-stage method) has been introduced in kit form by several commercial suppliers. We have been closely involved with the assessment of this new methodology with regard to its applicability to the potency measurements of different FVIII materials (including plasma and high-purity concentrates) and Dr Barrowcliffe has recommended to the ISTH and the EP that this method replace the two-stage as the reference method. As part of our support for this move, we are currently developing our own in-house chromogenic assay, using highly-purified FIXa and Factor X (FX) prepared and filled at NIBSC.

In general we have shown good agreement with manufacturers' labelled potencies during this period, and because of long experience with BPL's 8Y product we have reduced testing frequency to one batch in 3. The more highly purified products, particularly those prepared by ion-exchange/solvent-detergent methods, have tended to show discrepancies between one-stage and two-stage or one-stage and chromogenic methods, and this problem has been intensively investigated (see Sections 1.1.1(c) and (f)). The discrepancies are not always consistent from batch to batch, and in some cases have led to relabelling. For instance, one batch of high-purity FVIII from Alpha was found to be around 60% of labelled potency by NIBSC (two-stage) assays - subsequent re-assessment by the manufacturer using the chromogenic method agreed with our figures, and the batch was re-labelled. The savings to the Department of Health on this one batch alone were approximately £250 000. We also advised the Scottish National Blood Transfusion Service (SNBTS) to change their assay methodology following low potencies found on initial pre-licensing batches of their high-purity product H8 - they have now switched to the chromogenic method and we have good agreement.

1.1.1 (c) Standardisation and Assay Methodology

At the time of the last SPAC review, the problems of potency assessment of very high-purity concentrates prepared by monoclonal antibody affinity chromatography had been recognised. When assayed against the existing WHO standard these products gave discrepancies between the various assay methods and large inter-laboratory variability with any one method. A preliminary

products was even worse.

An international workshop, attended by all major world manufacturers and several control authorities in the USA and Europe, was held at NIBSC in May 1990 to discuss these problems. Following this meeting, it was decided to establish a small working party to come up with recommendations for the following:

1) Standardised assay methodology for high-purity concentrates.

2) Appropriate standards and methods for recombinant concentrates.

Further research and development at NIBSC identified some technical features of the assay methods which were particularly important for high-purity and recombinant concentrates. Subsequently a collaborative study was organised in which samples of both recombinant products, 2 monoclonal products, and a conventional high-purity product were assayed against the 4th IS, an intermediate-purity product, using the standardised assay methodology developed at NIBSC. From the results of this study and further discussions of the Working Party the following conclusions were drawn:

i) Good agreement between laboratories in assays of the high-purity and recombinant concentrates against the intermediate-purity concentrate standard can be achieved within any one method by adopting standardised assay methodology.

ii) There is no need for a separate standard for recombinant FVIII.

iii) Since discrepancies between potencies by different methods still exist even when standardised methodology is used, it is necessary to decide on one of the 3 methods as a reference method.

iv) Since the chromogenic method gave the lowest inter-laboratory variability in this study, and is the most biochemically-based method, it should be adopted internationally as the reference method.

These conclusions were accepted by the Scientific and Standardisation Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) and the "Recommendations for assay of high-purity FVIII concentrates" are about to be published as an SSC document. The same recommendations were made by Dr Barrowcliffe to the EP, and following a large European collaborative study in which excellent results were obtained, have been accepted by the EP Commission (see Section 1.2).

1.1.1 (d) **Structural Studies**

FVIII Characterisation Methods

i) Immunoblotting: firstly, a programme of method optimisation was followed and then the usefulness of some 25 monoclonal antibodies to FVIII from various laboratories were assessed. Currently using polyclonal anti-peptide antibodies, immunoblotting can directly visualise most or all HC species present in FVIII concentrates with specific activities of 50-100 iu/mg or higher.

ii) SDS-immunoelectrophoresis: this method utilises the specific binding of affinity-purified ^{125}I-labelled anti-FVIII antibodies to FVIII in an SDS-PAGE system: the antibodies are derived from the plasma of haemophiliacs with inhibitors to infused FVIII. Having modified the technique from the original report, it was used to visualise FVIII heavy chains in a wide range of products (including recombinant). In addition, we directly compared the proteolysis of FVIII as seen in highly purified FVIII with that in plasma, showing the cleavages to be identical.

iii) Using a range of monoclonal antibodies directed against different epitopes on FVIII, a series of enzyme-linked immunosorbent assays (ELISAs) was set up. These are capable of quantifying the different FVIII polypeptides present in FVIII concentrates or plasmas, except that some combinations gave lack of parallelism in dose-response between plasma standards and highly-purified FVIII concentrates, making accurate quantitation difficult.

FVIII Product Characterisation

Running in parallel with the biological activity assays, the immunological methods above were used to characterise the FVIII in a range of products. Immunoblotting of the older low-purity concentrates gives poor results, however the high-purity and very high-purity products have been investigated. Use of polyclonal antibodies raised against synthetic FVIII peptides (see below) has enabled visualisation of FVIII heavy chain 50 kDa cleavage products in one HP concentrate prepared by chromatography.

The Weinstein technique is better suited to visualisation of FVIII heavy chains in the full range of products including both high- and low-purity concentrates: the newer monoclonal antibody-purified and recombinant FVIII appear free from fragments derived by cleavage of 90 kDa heavy chains in comparison to some older (mid 1980s) products.

Analysis by polypeptide-specific ELISAs suggested that all plasma-derived and one recombinant FVIII product contain roughly 1:1 ratios of heavy and light chains: in addition all contain a mixture of heavy chains of varying lengths due to partial removal of the heavily glycosated B domain of the protein.

Comparison between Plasma-Derived and Recombinant FVIII

Our studies described earlier revealed that, unlike licensed pdFVIII products, for full activity in coagulation assays rVIII required predilution in severe haemophilic plasma: however this is not an altered characteristic of the rVIII molecule itself in comparison to pdVIII since Xa generation studies with a purified chromogenic system showed this effect to be mainly due to absence of von Willebrand factor in rVIII.

Following purification of pdVIII and rVIII, immunoblotting and SDS-PAGE with silver staining structural characterisation of rVIII showed the same species of heavy and light chains to be present in rVIII and pdVIII, although the spectrum of heavy chains larger than 90 kDa was different. Treatment of both pdVIII and rVIII by the natural activator thrombin also gave identical cleavage patterns indicating normal activation of the recombinant molecule. Finally, polypeptide-specific ELISAs showed there to be a similar ratio of heavy chains to light chains in one rVIII product as in pdVIII, although the overall ratio of rVIII antigen to clotting activity was higher than for pdVIII, indicating either expression of some inactive molecules or perhaps that differences in glycosylation affect biological activity.

Antibodies to Synthetic Peptides (This is a collaborative project with Dr D S Pepper at the SNBTS National Science Laboratory)

To study the functional importance of an unusual highly acidic linker region (between the A_1 and A_2 domains) synthetic peptides corresponding to portions of the sequence were prepared and used to immunise rabbits and chickens. The resulting antibodies showed several interesting characteristics: a) all the chicken anti-peptide antibodies were inhibitory to human FVIII activity *in vitro*, but not the rabbit antibody - indicating species differences probably related to the level of conservation between human and rabbit or chicken proteins; b) inhibitory antibodies raised against non-overlapping peptides showed synergistic activity against FVIII indicating at least 2 distinct functionally important areas; c) chicken antibodies derived from eggs of immunised hens can be produced easily in large quantities and make excellent immunological reagents.

1.1.1 (e) Immunological Studies

Aside from the issue of viral safety, the 2 consequences of FVIII therapy which are of most clinical concern are the possible deleterious effects on the immune system, and the development of antibodies to FVIII in recipients. The Division has been carrying out *in vitro* studies which are relevant to both these important aspects.

Immunosuppressive Actions of Concentrates (in collaboration with Division of Immunobiology)

Evidence has accumulated that long-term FVIII therapy is associated with

impairment of the cellular immune system in HIV negative haemophiliacs, manifested by abnormal T-lymphocyte and monocyte behaviour, and decreased skin antigen responses. The degree of immunosuppression is much less than in HIV disease, and its clinical significance is uncertain, though it has been suggested that haemophiliacs have increased risk of infection. The mechanism is unknown but it is assumed that repeated infusion of the large amounts of non-FVIII proteins is in some way responsible. Of perhaps greater concern is the potential immunosuppressive effects of concentrates in HIV positive patients, which could conceivably hasten progression to AIDS.

In vitro studies of immunosuppression had been initiated at the time of the last SPAC review, and have since been greatly extended with the help of a grant from the SNBTS. These studies are carried out in collaboration with Dr Robin Thorpe, Dr Meenu Wadhwa and Dr Anthony Mire-Sluis in the Divison of Immunobiology. Studies have continued to focus on inhibition of interleukin-2 (IL-2) secretion and it was shown that the inhibition of IL-2 secretion observed with various concentrates after incubation *in vitro* with T cells was not an *in vitro* artefact, since it was also found *in vivo* in a mouse model. A particularly interesting finding was that all high-purity concentrates prepared by conventional chromatography displayed significant inhibition of IL-2 secretion, in contrast with concentrates purified by monoclonal antibody chromatography which showed no inhibition of IL-2 secretion. These data suggest that a minor contaminant, rather than the bulk protein level, is responsible for the immunosuppressive actions.

Most recently, a contaminant has been identified which appears to be responsible for most of the *in vitro* effects; this is transforming growth factor-beta (TGFß), a cytokine present in all blood cells but predominantly in platelets. Antibodies to TGFß abrogated most of the immunosuppressive effects of concentrates, and TGFß levels measured by biological and immunological assays correlated well with immunosuppressive activity. In particular, high levels of TGFß were found in high-purity concentrates purified by conventional chromatography, but none was found in monoclonal antibody purified concentrates. A particularly interesting aspect of the biological activities of TGFß is its selective inhibitory action on CD4 cells, which may be relevant to the clinical trial data on CD4 levels with various concentrates - our data could explain the findings that high-purity concentrates prepared by monoclonal antibody chromatography, but not those prepared by conventional chromatography, may have less inhibitory action on CD4 cells than intermediate-purity products. However, our results also emphasise that intermediate-purity concentrates differ widely in TGFß content - since TGFß originates in platelets this could be due to differences in platelet contamination and subsequent handling of starting plasma. Attention to this aspect of plasma for fractionation could therefore yield rich dividends in production of less immunosuppressive concentrates.

Clinical Immunogenicity

The development of antibodies to FVIII occurs in approximately 15% of haemophiliacs, and currently there is intensive debate as to whether the incidence has increased with the introduction of new concentrates, especially those produced by monoclonal antibody and recombinant technology. Efforts by manufacturers to assess possible altered antigenicity of their products have involved various animal models none of which satisfactorily predicts the human situation. Although it is very difficult to design relevant *in vitro* studies in this area, we have attempted to address this problem from 3 points of view:- studies of antibodies from patients with FVIII inhibitors; generation of human antibodies *in vitro* by recombinant phage technology; development of a test for activation of FVIII which may be relevant to altered immunogenicity (see Section 1.1.1.(f)).

i) Human Antibodies from Patients

A panel of antibodies from patients was investigated for their ability to inhibit various kinds of FVIII concentrate. It was found that the antibody titres were approximately 4 times higher with a monoclonal antibody purified concentrate than with an intermediate-purity product - these results have implications for treatment of inhibitor patients with various products. It is intended to obtain further samples of antibodies from patients treated with recombinant FVIII alone, to test the reactivity of these antibodies with various types of FVIII.

ii) Human Antibodies *in vitro* by Recombinant Phage Technology

With the arrival of Dr Ouwehand in the Division, the opportunity arose to collaborate in the development of anti-rVIII human mabs using the recombinant phage technology developed by Dr Greg Winter's group in Cambridge. Such human mabs may be used in studies of the immunogenicity of recombinant FVIII.

We have investigated whether the immunoglobulin variable domain gene repertoire derived from the RNA of peripheral blood lymphocytes of normal healthy individuals is FVIII reactive. For this purpose the V gene repertoire of heavy and light chain (VH and VL respectively) was expressed on the surface of filamentous phage as single chain variable domain fragments (scFv's).

By several rounds of phagemid selection, using rFVIII as antigen three rFVIII binders were obtained. VH and VL gene sequencing revealed 2 unique clones and in one of the 3 clones the VL domain was absent. The specificity of the FVIII antibody fragments was analysed in an ELISA with a panel of 13 protein antigens and in flow cytometry by measuring binding to peripheral blood cells.

The scFv's of the 2 clones H2 and D3 which were identical on sequence analysis were both rFVIII specific and precipitated the light chain of plasma derived FVIII. The binding of scFv H2 to rFVIII was inhibited by 10 of the 20

plasmas from patients with FVIII inhibitors and by 3 of the 5 plasmas from patients with FVIII autoantibodies.

The heavy chain only antibody (dAb) produced by clone C8 showed besides the FVIII reactivity also reactivity with human IgG and IgM. Further studies are in progress to investigate the structural features of the VL domains which are responsible for this cross-reactivity.

We are now also establishing a V gene phage display library from the RNA of B lymphocytes of a haemophilia patient with a high level of FVIII inhibitors. To this end FVIII specific B cells were selected from lymphocytes by adherence to rFVIII. It is assumed that multiple FVIII scFv's with high affinity will be obtained in this manner. These scFv's will be characterised by V gene derivation and level of somatic mutation, for their capacity to inhibit FVIII and by immunobiochemical methods the epitopes recognised by the scFv's will be characterised.

Human anti-idiotype antibodies will be selected from V gene expression library against the anti-FVIII scFv's and these anti-idiotype antibodies will be used to analyse the structural features of the B cell repertoire of the initial patient and of other patients with FVIII inhibitory antibodies.

1.1.1 (f) Activation of FVIII in Concentrates

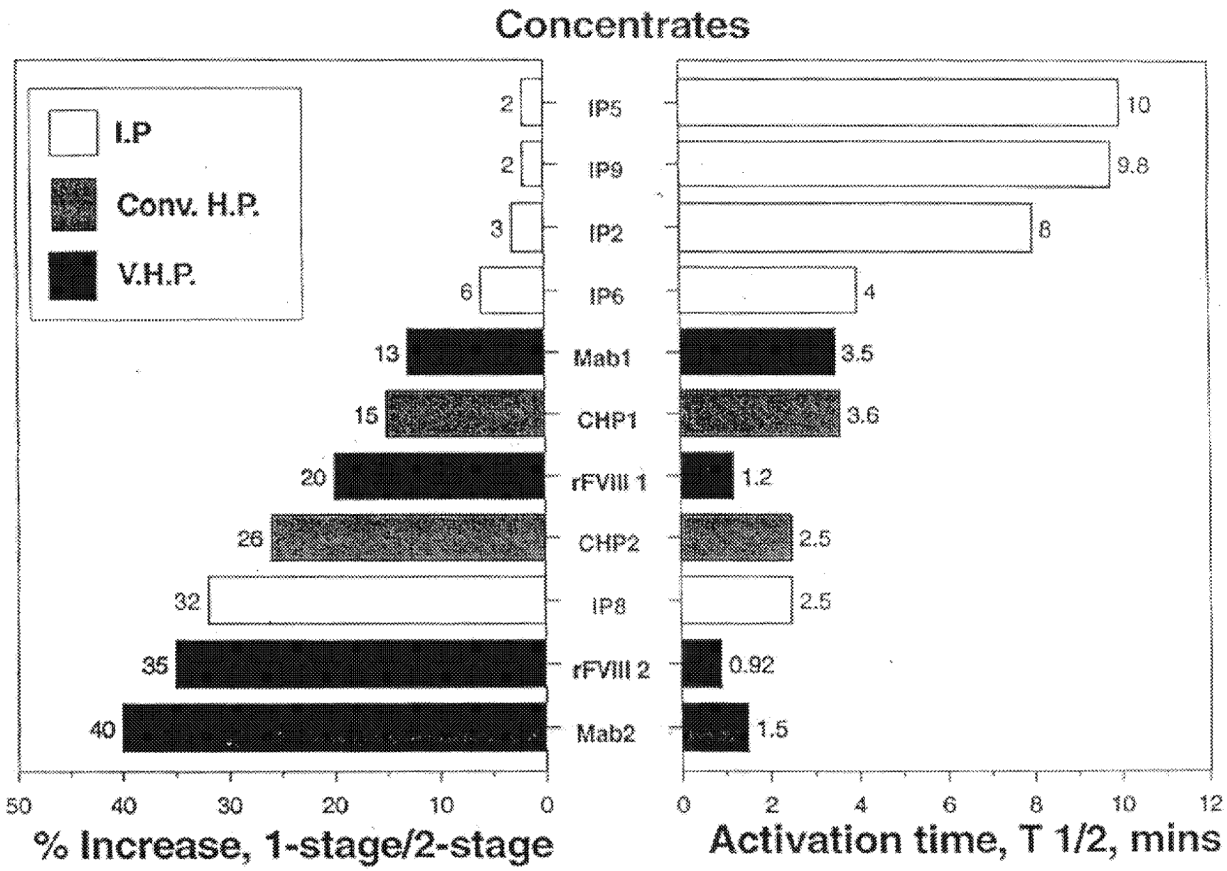
There is a general trend for many of the current products, particularly those of high-purity, to give discrepancies in potencies between the 2 major clotting methods, the one-stage and the two-stage. In a survey of data on 13 concentrates from our Division, 9 showed higher potencies in one-stage assays than by the two-stage method, by amounts ranging from 8-40%. A possible explanation could be the presence of small amounts of activated FVIII, which increases its potency in one-stage but not in two-stage assays.

In vitro Studies

The *in vitro* system which had just been developed at the time of the last SPAC review has been further refined, and extended to study the whole range of FVIII products. In this system the speed of generation of Factor Xa (FXa), measured by T½, the time to half-maximal enzyme concentration, is an index of possible activation, though other influences on the rate of Xa generation are also recognised.

In general it was found that the more highly purified preparations were associated with more rapid Xa generation rates, and the recombinant concentrates gave the most rapid rates of all. There was a very good correlation between T½ measurements in this system and one-stage/two-stage discrepancies, as indicated in Figure 1. More recently it has been found that the presence of von Willebrand Factor (vWF) and/or phospholipid (PL) in concentrates also exerts a major influence on Xa generation rates, and current

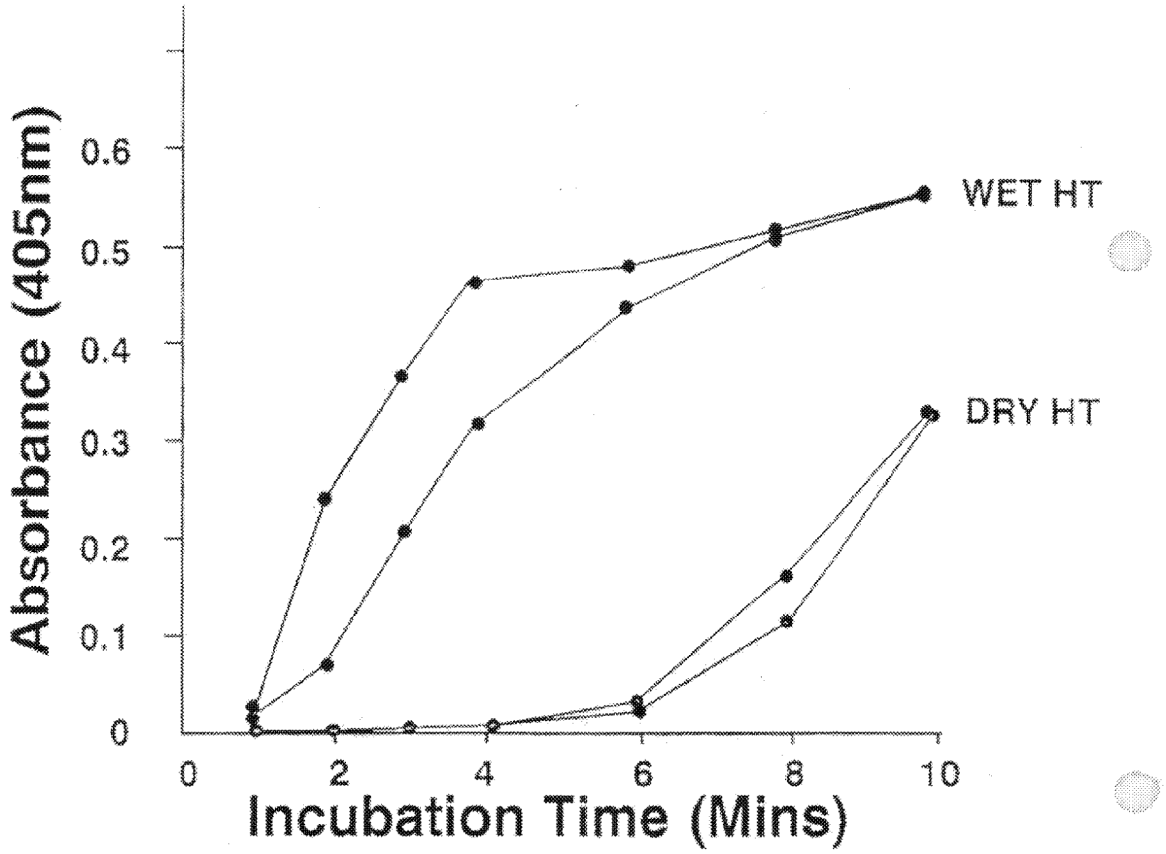
Figure 1 Comparison of one-stage/two-stage potencies of Factor VIII concentrates with their activities in a Factor Xa generating system.



T½ = time to half-maximal factor Xa concentration. The concentrates with the greatest discrepancies between the assays had the most rapid factor Xa generation.

IP = Intermediate purity
CHP = Conventional high-purity
Mab = Monoclonal antibody purified
rFVIII = Recombinant factor VIII

Figure 2 Factor Xa generation with CPS Factor VIII concentrates subjected to dry or wet heat.



studies are directed towards determination of the relative importance of activated FVIII, vWF and PL for each of the products.

A particularly interesting finding was our results on 2 intermediate-purity concentrates prepared by the Dutch Red Cross, which differ only in the method of viral inactivation - dry heat and wet heat (pasteurisation). Following the replacement of the dry heated product with the pasteurised product in Holland and Belgium there was an increased incidence of antibodies to FVIII in recipients and the pasteurised product was withdrawn. Our data (Fig 2) showed a more rapid rate of FXa generation for the wet heated than the dry heated product, indicating the likely presence of activated FVIII. It is possible that the presence of activated FVIII could account for the increased development of antibodies with this concentrate, and if so our *in vitro* test could be a useful marker for manufacturers to test for possible changes in antigenicity. In fact we have already been asked by 2 manufacturers to test their products before and after the introduction of a heating step to solvent/detergent inactivated products.

In vivo Studies

In collaboration with Dr Janet Littlewood at the Animal Health Trust, Newmarket, and supported by a grant from BPL we have investigated *in vivo* effects of activated FVIII in a haemophilic dog model. The haemostatic effect of FVIII was assessed with or without prior thrombin activation, using the cuticle bleeding time. Porcine FVIII was used for these studies since it is less immunogenic than human FVIII and the thrombin activated form is more stable.

The results showed that despite a 4-fold increase in activity *in vitro* by the one-stage assay after thrombin activation, there was no comparable increase in haemostatic effectiveness. These results imply that the higher one-stage potencies in some human concentrates are unlikely to be predictive of their clinical efficacy and that the two-stage or chromogenic method should continue to be the reference method for labelling of potency.

1.1.2 Factor IX Concentrates

(a) **Product Characteristics**

Previously, FIX deficiency has been successfully treated with materials known as 'prothrombin complex concentrates' (PCCs) containing roughly equal activities of Factors II (FII) (prothrombin), IX and X. There has been, however, a history of infrequent episodes of thrombotic episodes (sometimes fatal) connected with treatment, and concentrate suppliers have responded to the situation basically by 2 related routes:

(i) production of 'single-component' FIX-only concentrates which have been shown to be less thrombogenic in animal models.

(ii) monitoring the levels of activated clotting factors (thrombin, FXa and

especially FIXa) in processing and in the final products.

In May 1992 an international workshop was held at NIBSC to discuss this issue and in particular to evaluate testing procedures on both products and patients' plasma after injection. From this workshop proposals were made to standardise testing procedures for activated clotting factors, particularly in the new high-purity concentrates.

(b) **Batch Control**

Potency and *in vitro* thrombogenicity tests are carried out on all batches. In the beginning of the review period potency discrepancies were found with some batches from BPL, but these were resolved when BPL changed from the two-stage to the one-stage assay. Some batches of the SNBTS product have given borderline results in the EP thrombogenicity tests, but none have actually failed. We have continued to manufacture our own FIX deficient plasma for potency assays, using our own monoclonal antibody for immunodepletion.

Table 3 shows the numbers of batches of product received during the period under review, and highlights the move towards high-purity (HP) monocomponent products and, in 1993, the appearance of commercial non-UK HP batches on the market (previously the UK had been self-sufficient in FIX).

Table 3 FIX products submitted to NIBSC for batch testing

	Year			
	1990	**1991**	**1992**	**1993 (projected)**
NHS FIX (No of batches)	39	39	25	24
Commercial FIX (No of batches)	0	0	0	7
Commercial FIX (% of total)	0%	0%	0%	23%
Total No of batches submitted	39	39	25	31
High-purity batches (% of total)	0%	5%	20%	46%

Following investigative work in our laboratory in collaboration with the FDA it was found that good agreement on potency of several high-purity FIX products could be achieved using the existing International Standard (a PCC product) - there was no need for a separate standard for high-purity FIX.

(c) **Development of Thrombogenicity Tests**

Although the high-purity FIX concentrates have generally been less thrombogenic than PCC's in animal studies, developmental work by several manufacturers showed that some batches of HP FIX could give a significant thrombotic response in animals and this was not correlated with the results of existing *in vitro* thrombogenicity tests. Consequently some manufacturers are carrying out *in vivo* tests for thrombogenicity for batch control, and there is a clear need for an *in vitro* test which does correlate with the *in vivo* results for these newer concentrates.

We have developed a new assay for FIXa which gives promising results in this area. We focused on FIXa because it is known to be highly thrombogenic and because it seemed possible that some activation of FIX might occur during purification and viral inactivation. A highly-purified preparation of FIXa was made as a potential reference material, and detectable levels of FIXa were found in almost all high-purity concentrates, though PCC's could not be satisfactorily assayed because of interference from the other clotting factors.

An *in vivo* model for testing thrombogenicity of FIX concentrates was developed by Dr Gray using the rabbit Wessler stasis model established for antithrombotic drugs. Highly purified FIXa was extremely thrombogenic in this model and most of the high-purity FIX products showed some *in vivo* thrombogenic activity, though at a lower level than PCC's. There was a good correlation between FIXa concentrations and *in vivo* thrombogenicity.

This work aroused considerable interest when presented at the international workshop and we have had many requests from manufacturers for the FIXa assay methodology. Following discussions at the ISTH/SSC we have agreed to make a standard for FIXa and work on this project has commenced.

1.1.3 <u>Factor VII/Factor VIIa</u>

Although genetic deficiency of FVII is very rare, FVII concentrates are manufactured for treatment of these patients and are also used occasionally as a supplement to FII-IX-X concentrates for reversal of oral anticoagulant overdosage. FVIIa concentrates have given promising results in treatment of haemophiliacs with antibodies to FVIII, and products are under clinical trial from 2 manufacturers. In addition there is intense interest in measurement and molecular genetics of FVII since Professor Meade's pioneering work showing that high levels are strongly predictive of ischaemic heart disease.

1.1.3 (a) **Factor VII Concentrates**

Only one FVII concentrate product is available in the UK - this is manufactured by the BPL, Elstree. Control of this product (approximately 6 batches per year) involves the testing of plasma pools and product for viral markers and testing of final product for potency (FVII clotting activity).

Initial potency estimations were carried out at NIBSC to check the manufacturer's labelled potency by FVII clotting assay, relative to the 1st IS Factors II, VII, IX, X, plasma, 84/665. We were concerned to find that our potency estimates indicated that the product was underlabelled by approximately 50%. Our assays on the manufacturer's house standard revealed that this was also apparently underlabelled to the same extent. Potency estimates carried out on samples of product by 2 independent laboratories agreed with the results of NIBSC. It was decided that the best way to resolve the discrepancy would be to calibrate an ampouled, freeze-dried preparation of the product which could then be used as a reference preparation for potency estimation by both NIBSC and the manufacturer.

A collaborative study was organised in which 8 UK laboratories assayed the candidate ampouled FVII concentrate (89/590) against the 1st IS plasma standard (84/665). It was interesting to note, from this study, that the manufacturer's potency estimate (14.2 iu/ml) showed the same discrepancy with that of NIBSC (22.5 iu/ml) as was found in earlier assays of the product. However, the NIBSC potency agreed well with the other participating laboratories and the overall combined mean potency (20.4 iu/ml); the reason for the manufacturer's low potency estimate was unclear. However, the manufacturer agreed to accept the overall combined mean potency and the ampouled concentrate was established as the 1st BS, FVII, Concentrate (89/590). We have experienced excellent agreement, with the manufacturer, in potency estimation since the adoption of the 1st BS FVII, Concentrate (89/590).

1.1.3 (b) Standardisation of Factor VIIa Concentrates

Activated factor VII (FVIIa) concentrates are undergoing clinical trials for the treatment of haemophiliacs with inhibitors to FVIII and FIX. The FVIIa concentrates currently available are derived either from recombinant DNA technology with the human gene expressed in baby hamster kidney cells (Novo Nordisk A/S, Denmark) or purified from plasma (CNTS, France). Comparison of the dosage and hence the clinical effectiveness of these 2 products is complicated by the administration of one product by weight and the other in terms of "units" of FVII clotting activity.

An international collaborative study was organised in order to investigate and compare the assay of ampouled preparations of both recombinant (rFVIIa) and plasma-derived (pFVIIa) FVIIa concentrates against the 1st IS plasma standard (84/665) with the objective of calibrating an international reference preparation. Assays were carried out according to defined assay conditions, established after research and development in the Division, and using reagents supplied specifically for the study (thromboplastin and FVII-deficient plasma).

Analysis of the raw data indicated some degree of non-parallelism between the log dose-response relationships of the FVIIa concentrates and the 1st IS plasma (84/665) as might be expected from the comparison of "unlike" materials,

however, the 2 FVIIa concentrates were parallel to each other. The validity of using a calibrated reference preparation consisting of one type of FVIIa concentrate (eg recombinant) for the potency estimation of the second type of concentrate was supported by the parallelism of the log dose-response relationships of the 2 concentrates and also from the good interlaboratory agreement in potency estimates (gcv 7%) for the plasma FVIIa concentrate when they were recalculated using the recombinant FVIIa concentrate as reference material (Fig 3). The use of a FVIIa concentrate reference preparation would therefore lead to much closer agreement in potency estimates between laboratories compared to the use of the 1st IS plasma (84/665).

It was therefore proposed that the recombinant FVIIa concentrate (89/688) be accepted as the 1st IS FVIIa Concentrate. This proposal was submitted to the Expert Committee on Biological Standardisation (ECBS) of the World Health Organisation (WHO) together with evidence of suitable stability of the ampouled preparation. The 1st IS Factor VIIa concentrate (89/688) was established in October 1993 with an assigned potency of 5130 iu per ml.

1.1.3 (c) Factor VII and phospholipids

The conclusion, by others, that the increased FVII activity seen in men at risk of cardiovascular disease (CVD) was due to a novel form of activated FVII, complexed with phospholipid, was based on the observation that phospholipase C (PLC) reduced, *in vitro*, the FVII clotting activity in plasma from men at risk of CVD. It was proposed that PLC cleaved the FVII-phospholipid complex to release normal zymogen FVII. Our own findings were not consistent with this hypothesis and we concluded that the PLC-mediated reduction in FVII activity was caused by inhibition of FVII. The "PLC effect" appears to be no more than an *in vitro* artefact and we concluded that its measurement would be no more useful in predicting a risk of CVD than measuring plasma triglyceride levels.

1.1.3 (d) Molecular Biology of FVII

This is a collaborative project with Dr E G D Tuddenham and colleagues of the Haemostasis Research Group at Northwick Park Hospital, and has been largely carried out by Dr G Kemball-Cook during his one-year secondment to Northwick Park.

The project has involved:

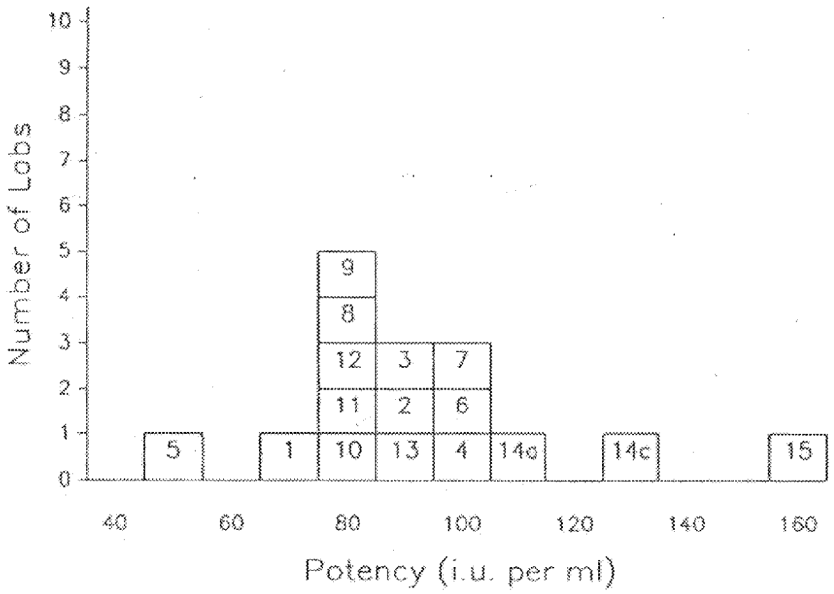
1) the construction of a high-efficiency mammalian cell expression system in Chinese hamster ovary (CHO) cells for production of wild-type and mutant FVII.

2) identification of naturally occurring dysfunctional mutants (in human subjects) of the coagulation protein FVII.

Figure 3 The effect of different reference preparations on the agreement between laboratories in the potency estimation of plasma-derived FVIIa concentrate.

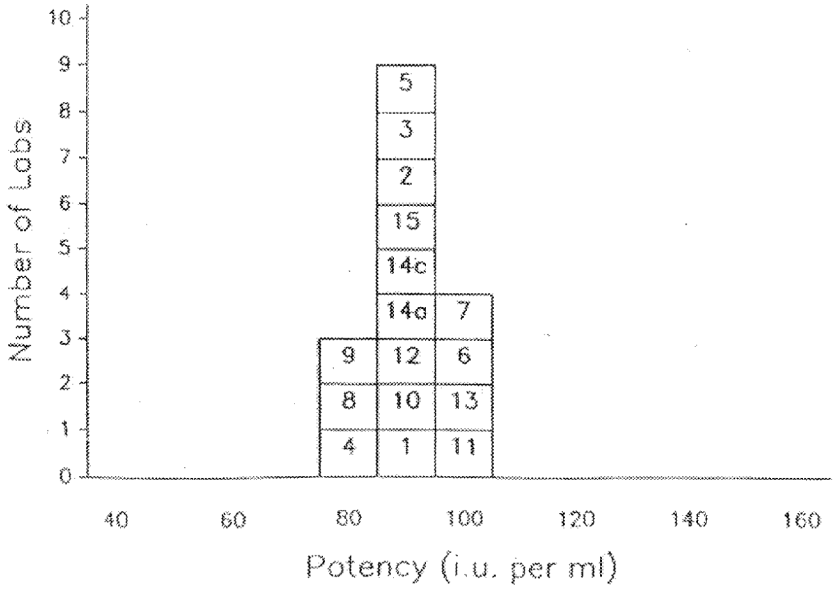
a) relative to the 1st IS Factors II, VII, IX, X, plasma (84/665)

Interlaboratory variability (gcv) 29.7%



b) relative to the 1st IS Factor VIIa, concentrate (89/688)

Interlaboratory variability (gcv) 7.0%



3) sequencing of the FVII gene from these subjects to identify the causative mutation(s).

4) resynthesis of the mutant DNA by site-directed mutagenesis of the wild-type FVII cDNA, and finally

5) expression of wild-type and recombinant mutant FVII molecules using a high-level mammalian cell expression system.

The construction of the mammalian expression vector pNeo1G502 and the development of the expression system in CHO cells was followed by production of large amounts of wild-type and mutant FVII ready for purification. Altogether about 150 000 units of wild-type FVII were produced for purification and characterisation. Sequencing of the N-termini of the full-length and activated proteins gave identical primary structure to the human protein. The intention is that further work in this area will provide enough material to attempt X-ray crystallographic studies.

Three missense mutations in human subjects were investigated which are presumed to cause the dysfunction found in these patients' plasma. Following resynthesis of the mutations by site-directed mutagenesis of the wild-type DNA and insertion of the DNAs into the expression vector, large amounts of the mutants were produced for characterisation. Currently, one of the mutants has been extensively characterised in its interaction with tissue factor (the natural cofactor for FVII during coagulation), and the molecular cause of the factor's dysfunction is being assessed by use of the new technique, biospecific interaction analysis.

Now that Dr Kemball-Cook has completed his secondment period, it is intended to use similar techniques to express functionally important domains of the FVIII molecule.

1.1.4 Antithrombin III

Concentrates of this major coagulation inhibitor are used to treat patients with congenital deficiencies, though unlike haemophilia regular treatment is not required so the amount used is much less than that of FVIII. Concentrates are also used for prevention of endotoxin-induced disseminated intravascular coagulation in septic shock, though their clinical value in this condition is unproven. AT III is also the plasma protein through which heparin exerts its action, and studies of the heparin/AT III interaction are important for understanding the biological activities of both components.

1.1.4 (a) Control and Standardisation of Concentrates

An IS for AT III in plasma has existed since 1980. However, as is the case with most clotting factors, this is not ideal for assay of concentrates because of high variability between laboratories and between assay methods, and after

in house assessments, AT III concentrates from 4 manufacturers were compared against the plasma standard and against each other in an international collaborative study. The results showed much better agreement on potency with a concentrate as standard than with the existing plasma standard - accordingly one of the 4 preparations was established by WHO as the IS for AT III, Concentrate in October 1990.

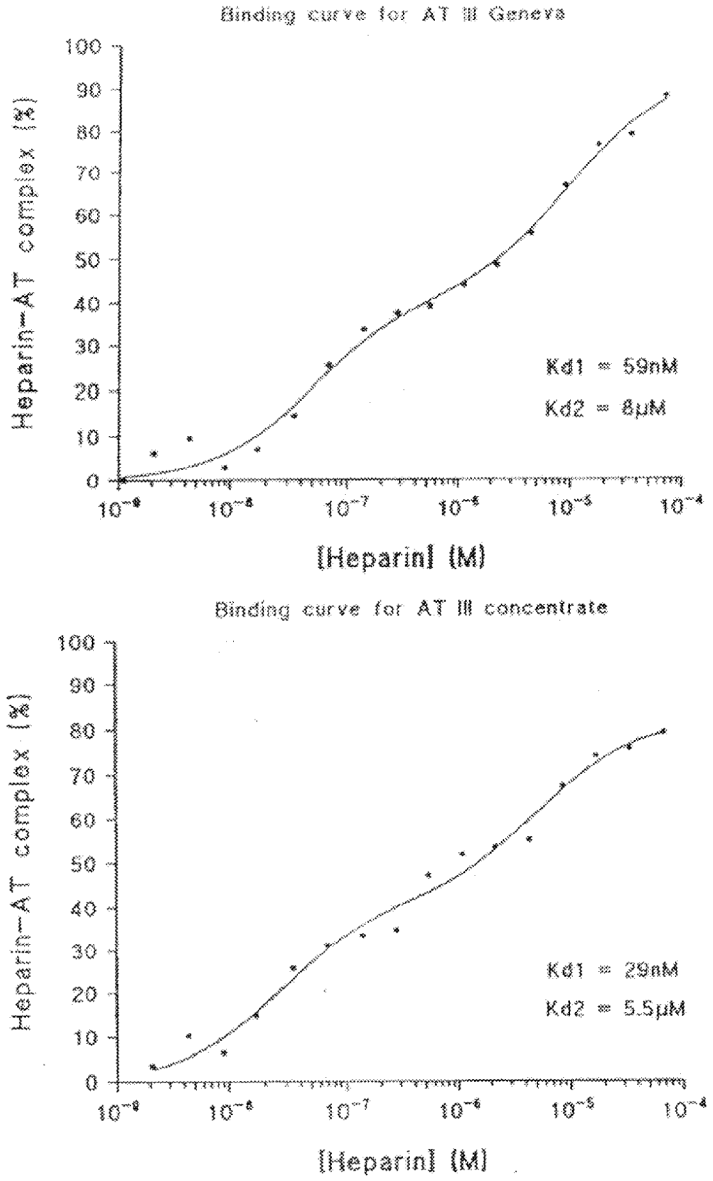
Two manufacturers (Kabi and BPL) are licensed in the UK, but samples are received only from BPL (approximately 6 batches/year). Potency assays have been satisfactory but discrepancies were found in assessment of the heparin binding fraction, which is normally done by a semi-quantitative crossed immunoelectrophoresis method. Our results on several batches indicated amounts close to the EP minimum limit of 60% (BPL approximately 80%). Although this method is difficult to standardise, our results were backed up by similar data from heparin affinity chromatography and from a new monoclonal antibody technique (see below). Following meetings with the manufacturers they agreed to change their methodology and since then much closer agreement has been found. However, since the proportion of non-heparin-binding material continues to remain high, the manufacturers are now investigating changes in their production method to minimise the proportion of this fraction.

1.1.4 (b) Studies of Interaction of AT III and Heparin

Antithrombin III is a serine protease inhibitor primarily of thrombin. However, inhibition develops only slowly in the absence of heparin or related glycosaminoglycans. These molecules are believed to bring about the dramatic rate enhancements of binding of thrombin and AT III by several mechanisms: by binding to both proteins together, increasing the probability of reaction (template mechanism); by inducing conformational changes in one or both proteins; and by neutralising positive charges (heparins are sulphated) on the protein surface which otherwise repel. AT III has a higher affinity for heparin than thrombin and studies over the last few years have identified the amino acid residues that make up the heparin binding site. Recently we have generated a monoclonal antibody against the AT III heparin binding site which has been used to develop an IRMA system capable of discriminating between normal AT III and AT III with abnormal heparin affinities. With the help of Dr Longstaff this system has been adapted to provide quantitative results for heparin - AT III affinities. By satisfying certain conditions in this assay it is possible to use it to determine indirectly apparent K_d values for heparin binding.

Subsequently, a set of binding equations was developed which generate isotherms to fit observed patterns of experimental data. These calculations gave K_d values for the heparin AT III interaction which have been used to measure precisely, for the first time, heparin affinity for AT III in its native environment (plasma). Furthermore, plasmas from patients with heterozygous populations of normal and mutant AT III have been studied to determine dissociation constants for heparin with normal and mutant inhibitor and their relative proportions in plasma, without need for prior purification. Hence we are

Figure 4 Studies of AT III/heparin binding in plasmas and concentrates



The AT III complexed by the monoclonal antibody on the plates decreases in the presence of increasing concentrations of heparin, as the AT III binds preferentially to heparin. The amount of AT III bound to heparin can be calculated and this is plotted against the heparin concentration.

Both the AT III concentrate and plasma from a patient heterozygous for a mutation affecting the heparin binding site of AT III (AT III Geneva) can be seen to contain 2 populations of AT III with different affinities for heparin. The dissociation constants for both populations can be determined from the binding curves.

able to get quantitative data on how a range of mutations around the AT III molecule affect heparin binding properties which relate to defective AT III function in vivo. The approach is also useful for investigating normal, denatured and proteolytically modified inhibitor proteins, which occur to variable extents in control batches of AT III concentrates. Figure 4 shows the detection and quantitation of 2 populations of AT III molecules, differing in their heparin affinities, in samples from a patient with a genetic mutation, and from an AT III concentrate.

These studies will be extended in the future to examine more forms of AT III (more mutants and complexed forms), and to include other types of heparin, including low molecular weight heparin, and related materials. This is a powerful experimental approach, providing precise quantitative data which can be linked to structure/function relationships in this interesting serpin.

1.1.5 Factor XI

Factor XI (FXI) deficiency is at least as common as FIX deficiency but many patients have only mild clinical symptoms. Others however, bleed more severely and require treatment after trauma or operative procedures. The main concern with concentrates of FXI is their potential thrombogenicity.

In a collaborative project partly funded by BPL, Elstree, we have assessed the thrombogenic potential of FXI concentrate in the rabbit model used for FIX concentrates. This product is in the developmental/clinical trial stage, and it is prescribed on a named patient basis. BPL is one of the 2 manufacturers of FXI concentrate in the world and they are in the process of obtaining an IND in the USA. Activated FXI is known to be highly thrombogenic and this product theoretically carries much higher thrombotic risk than the high-purity FIX concentrates. The only *in vitro* thrombogenicity test carried out on this concentrate is the non-activated partial thromboplastin time which does not appear to correlate with *in vivo* experimental thrombosis. We have investigated the effect of addition of varying concentrations of heparin on the thrombogenic potential of FXI. At least 10 batches of FXI concentrate have been studied and in order to overcome the varying level of platelet factor 4, at least 10 iu/ml of heparin were required to ensure non-thrombogenicity in all the batches. Based on these results, Bio Products Laboratory is now routinely adding 10 iu/ml of heparin to the final formulation of their FXI concentrate. A new *in vitro* test for FXIa has been developed which shows a promising correlation with the *in vivo* results. Currently all batches manufactured are monitored for thrombogenicity by BPL and NIBSC, using both *in vivo* and *in vitro* methods.

1.1.6 Factor XIII

Concentrates of FXIII are manufactured by BPL and by Hoechst for treatment of the few congenitally deficient patients and are sometimes used to promote wound healing. The Hoechst material is made from placentae and no further batches will be released as it will be superseded shortly by a plasma derived

material. Aside from the issue of viral safety there are no major concerns with this product and no control testing is carried out in the Division.

1.2 STANDARDS FOR CLOTTING FACTORS

The Divisions's work on standards complements its control activities by ensuring that manufacturers and clinical laboratories all have access to the appropriate standards calibrated in International Units. As a result of work initiated during the late 1970's and continued during the 1980's, it became clear that 2 separate types of standard are required for most clotting factors - concentrate standards for control of therapeutic materials, and plasma standards for assay of patients' plasma samples. In addition to the establishment of International Standards through WHO, the Division is also committed to the supply of Working Standards in large quantities to UK manufacturers and clinical laboratories, and has recently been heavily involved in the provision of Working Standards to the European Pharmacopoeia.

The philosophy of the Division is to promote the use of these standards, and hence of International Units, throughout the world by meeting whatever demand is necessary and by the use of appropriate publicity. Of the 5 most heavily used International Standards in the Institute, 3 are for clotting factors (1st, 3rd and 5th), and of the 4 most heavily used non-International preparations, British Standards for clotting factors are 1st, 2nd and 4th, the 3rd being an anti-D reagent. A brochure was prepared for the Congress of the International Society on Thrombosis and Haemostasis and distributed to all 4,000 delegates (copy appended to this report). A similar brochure on standards in Transfusion Medicine is also included. This policy has been successful in that all manufacturers of clotting factor concentrates use the WHO standards as their primary standards, and the supply of National Working Standards, initiated by the Division in 1976, has now been copied in the USA, Europe and other countries.

1.2.1 International Standards

During the review period, new International Standards have been established for α-thrombin, plasma fibrinogen, antithrombin III concentrate and Factor VIIa concentrate, and replacements have been established for Factor VIII/von Willebrand Factor in plasma and Factor II, IX, X concentrate. Each of these has involved the organisation of a major international collaborative study, except the II, IX, X concentrate, where a small study was carried out to check a material previously calibrated. Development work has been carried out on a replacement for the FVIII concentrate standard, and work will shortly be initiated on development of a standard for FXI. Work on standards for antithrombotic drugs, thrombolytic agents and transfusion medicine is described in Sections 2, 3 and 4 respectively.

1.2.2 British Standards

These are issued to manufacturers of concentrates, and to hospital and transfusion centre laboratories for direct use as working standards in assays of products and patients' samples.

1.2.2. (a) **Concentrate Standards**

The BS for FVIII concentrate has been one of NIBSC's most heavily used standards for a number of years, with over 2,000 ampoules distributed per annum (mainly to the NHS blood product manufacturers in Elstree and Edinburgh). Since until relatively recently the maximum fill size available in the Standards Division has been 3,500 ampoules, the BS has needed frequent replacement (each time requiring a fresh collaborative study for calibration). In 1990 we carried out studies into the use of a new Paxall vial-filling machine with the capacity to fill 10 000 vials. The stability of both concentrate and plasma materials filled using this technology was satisfactory. Thus, in 1991 the 10th BS for FVIII Concentrate (10 000 vials) was calibrated and use began in 1992, with a projected lifetime of 4 years. Recently the 10th BS has undergone a recalibration involving a shift of 10% upwards from the original assigned potency: although the reason(s) for this apparent potency shift (when assayed against the current IS) are not known with certainty, it is likely that changes in FVIII two-stage methodology in the laboratories involved in the collaborative studies were responsible: such changes are part of the experience of standardisation of biological activities and serve to underline the vital importance of primary reference standards.

A similar concentrate working standard is issued for Factors II, IX, X and the 2nd Standard required replacement during 1993. Since this is used less frequently than the FVIII standard (~500 ampoules/year) a batch of 3,500 was made and established as the 3rd BS after a UK collaborative study.

1.2.2 (b) **Plasma Standards**

The BS FVIII plasma has been the most heavily used NIBSC standard for over 10 years and the take-up increases each year (approximately 4,000 per annum). The BS is now extensively used in Haemophilia Centres, General Hospital Haematology Departments and Blood Transfusion Centres all over the UK, as a working standard for any FVIII assay on plasma samples. As for the BS concentrate standard, the necessity for frequent replacement drove us to investigate the vialling option to increase the fill size to 10 000. Trial studies completed in 1990 indicated that plasma freeze-dried into vials in Standards Division would be stable and display excellent vial-to-vial reproducibility and so the 18th BS plasma was filled into 10 000 vials. Accelerated degradation studies on the 18th BS confirmed its stability, and it has performed very well over its 3-year lifetime (over 400 requests to over 100 laboratories). Recently the 18th BS has been superseded by the 19th BS, another 10 000-vial fill.

The BS for Blood Coagulation Factors, plasma is available, free of charge, to NHS laboratories for use as a daily working standard in the measurement of 7 different parameters: von Willebrand Factor (ristocetin cofactor), von Willebrand Factor (antigen), Factor IX, Antithrombin III, Factor VII, Protein C and Protein S antigen. This standard is also subject to heavy demand with over 3,400 ampoules despatched during 1992. Such heavy usage made it impractical to continue using ampoules for filling the replacement standards since the 4,000 x 1 ml capacity only satisfied the demand for approximately 15 months. With the current 5th BS Blood Coagulation Factors, Plasma it was therefore decided to switch to a 10 000 x 1 ml fill in rubber-capped vials. This has prolonged the lifetime of the standard to approximately 3 years and consequently also reduced the frequency of the recalibration exercise which involves 15 laboratories measuring 7 different parameters.

1.2.3 <u>European Standards</u>

1.2.3 (a) **European Pharmacopoeia (EP)**

During 1992 it emerged that the EP had obtained a substantial grant from the European Commission to establish a European Biological Standardisation Programme. This was done without consultation with NIBSC, although the Institute subsequently became involved in some of the projects. One of the first EP projects was the establishment of a European Working Standard for FVIII, Concentrate, and Dr Barrowcliffe was invited by the EP to be the project leader. As this involved a substantial amount of work for the Division a grant of £40 000 was negotiated. The project began in Summer 1992 and we were able to take advantage of our previous experience in FVIII standardisation to bring about a successful completion by May 1993, although because of a shortage of staff in Informatics the bulk of the statistical analysis work had to be contracted out. Market research had indicated a prospective demand of around 10 000 vials/year and therefore 2 batches each of 10 000 vials were prepared and are about to be shipped to the EP for distribution throughout Europe. If the anticipated demand materialises, this standard will require frequent replacement and it would be preferable to make larger batches; this would require either alterations to the Standards Division or contracting out and therefore needs to be considered in an Institute context.

During the latter half of 1993 Dr Barrowcliffe was asked to organise a similar project for the establishment of a European Standard for FIX, Concentrate; work on this will commence during 1994.

1.2.3 (b) **European Commission (EC)**

The EC's Bureau Communitaire de Rèference (BCR) was reorganised under the Measurements and Testing Programme. Although the BCR was mainly set up for physical and chemical standards, some biological materials including thromboplastins were established, and further proposals in the biological area were invited. Following meetings of European organisers of the National

External Quality Assessment Schemes (NEQAS) for coagulation testing, it was suggested that one or more reference plasmas for anticoagulant control should be prepared and NIBSC were asked to help. An advisory consortium was formed and Dr Barrowcliffe submitted proposals for a 3-year project to the EC's Measurements and Testing Programme. Funding was subsequently agreed and a grant of 227 000 ECU (Approximately £170 000) was awarded.

The project involves all the member states of the EC and the main objective is to produce reference plasmas which will aid the laboratory control of oral anticoagulant and heparin therapy, and in diagnosis and treatment of haemophilia.

The initial work is directed towards providing reference plasmas for the prothrombin time test which is carried out in hospital laboratories more often than any other clotting test and is primarily used to monitor the severity of oral anticoagulation. Although the present INR system for reporting the anticoagulant status of patients takes into account the variability caused by the use of different thromboplastin reagents there is still large interlaboratory variability in INR determinations. This variability may be caused by differences in the normal plasma which is used to calculate the patients' INR or by an incorrectly assigned ISI value on the thromboplastin reagent.

The present study is investigating the possibility of preparing stable freeze-dried normal and abnormal (anticoagulated) reference plasma preparations for the prothrombin time which resemble fresh plasma as closely as possible. It is planned to use such plasmas as reference materials in order to increase the agreement between laboratories. Since the project was initiated in June 1993 investigations have centred on the effect of freeze-drying and buffering on the stability of clotting factors and the prothrombin time of normal and abnormal plasmas. Trial fills are being carried out and candidate reference plasmas will be distributed to collaborating laboratories to determine their suitability under different conditions (reagents, apparatus) before major fills of 10 000 ampoules are undertaken.

1.3 HUMAN ALBUMIN SOLUTIONS

Batch control testing of human albumin solutions is centred on ensuring the safety of the product since its therapeutic efficacy in volume replacement cannot be assessed by the estimation of any potency. Over the last 4 years we have received samples from an average of 240 batches of albumin per year (41% from the BPL, Elstree; 44% from the SNBTS and 15% from commercial sources).

Although the plasma donations used to manufacture the albumin are all screened by the relevant BTC for HBsAg, anti-HIV 1/2 antibodies and anti-HCV antibodies, the plasma pools are again tested at NIBSC. Recently, testing of plasma pools has revealed several pools +ve for anti-HCV - this has largely resulted from the use of more sensitive test methods by NIBSC than were

originally used to screen the donors. The final albumin product undergoes pasteurisation (10 hours, 60°C) and is considered to be one of the safest blood-derived products in terms of viral transmission.

The final product and manufacturers' protocol are checked for conformity with the EP monograph. Some components which may cause adverse reactions in high concentrations (eg bacterial endotoxin, prekallikrein activator) and limits recently introduced to the EP monograph (eg aluminium) are also monitored.

1.3.1 Endotoxin

Although not strictly a pharmacopoeial requirement we test all batches of albumin for bacterial endotoxin (pyrogen) using the Limulus Amoebocyte Lysate Gelation test. Limits of 2.5 iu endotoxin per ml for 5% albumin solutions and 5 iu per ml for 20% albumin solutions, have been agreed between NIBSC and the manufacturers and these levels are rarely exceeded. However, in the past 3 years we have detected 7 batches which exceeded the limits and have subsequently not been recommended for release.

1.3.2 Prekallikrein Activator

Increased prekallikrein activator (PKA) levels are known to cause adverse, hypotensive, reactions during infusion of human albumin and consequently there is an EP limit of 35 iu per ml. Routine control testing, during 1991, revealed a large increase in the PKA content of human albumin solution from one manufacturer (Zenalb, BPL). Instead of the usual baseline values of <5 iu per ml we regularly found levels of 10 - 20 iu per ml and higher. One batch with a borderline PKA content was not recommended for release. The increase in PKA content of this product coincided with a change in the manufacturing process of the albumin. Apparently, an extra chromatographic step was introduced in order to reduce the aluminium content, however, this extra step also removed an inhibitor of PKA/kallikrein (the C1 esterase inhibitor) and so allowed PKA activation to proceed until stopped by the pasteurisation step. The manufacturer has subsequently modified the "in process" temperature in order to reduce the PKA activation and this has reduced the PKA levels overall although we still find the occasional batch with high levels (>10 iu per ml).

The introduction of the modified process also coincided with an increase in the number of reactions during infusion and Dr Barrowcliffe and Dr Hubbard from the Division were involved in meetings with the manufacturer and some clinicians to elucidate the causes of these reactions. At first it was thought that the increased PKA content may be the cause, however, not all of the implicated batches had high PKA levels. A second possibility was the reduced concentration of protease inhibitors particularly of C1 esterase inhibitor although there was no correlation between the levels in individual batches and the occurrence of clinical reactions. Another potential cause was the introduction of non-acid-washed filters, during manufacture, in place of acid-washed filters, which occurred at the same time as the new process was adopted. Although

there is no known reason why this should lead to reactions, the manufacturer has switched back to using the original acid-washed filters. Whatever the original causes the rate of reporting of adverse reactions has since diminished.

1.3.2 (a) **PKA Testing for the Western Province BTS, South Africa**

Following a request from this manufacturer in 1991, it was agreed to carry out this work at a cost of £550 per 6 samples tested. We have since received and tested 73 samples.

1.3.3 Aluminium

Concern over the risk of aluminium toxicity in patients with impaired or immature renal function prompted the introduction in 1991, to the European and British Pharmacopoeiae, of a statutory limit of 200 ug aluminium per litre of human albumin solution. Aluminium leaches out of the glass containers; this is a particular problem with paediatric doses as the small bottles have a high surface area, and levels can also increase on storage. Measurements of aluminium at NIBSC are not carried out as the Institute no longer has an atomic absorption spectrometer, and discussions are taking place with a number of external laboratories about possible contracting arrangements.

1.4 FUTURE DEVELOPMENTS

It is envisaged that the control work on albumin will continue at much the same level, subject to the current review. Work on FVII will diminish as the FVIIa standardisation and molecular biology projects have largely been completed. The fundamental work on AT III will be continued, as will the studies on FXI thrombogenicity, and some work will be initiated on development of FXI standards. The major new work envisaged is in the area of FVIII and FIX.

Both the FVIII and FIX product markets are in a state of rapid change. More highly purified products are becoming available, and in the case of FVIII, recombinant wild-type FVIII is likely to be licensed relatively soon: in addition, several biotechnology concerns are developing possible second-generation rFVIII (with engineered deletions) which may be offered for licensing within the next 2-4 years. Although FIX has not been produced for commercial purposes by recombinant technology, there is a continual drive to supply a cleaner, more pure product lacking any potentially thrombogenic activated factors. Thus, in both these areas there is a requirement for the Division to maintain and review our control and standardisation activities.

In the case of FVIII potency assessments, the collaborative study being organised for the replacement of the current IS, Concentrate will include at least one very high-purity material as a candidate standard, and close attention will be paid to the performance of the chromogenic assay for FVIII (the proposed new EP reference). Development of our in-house chromogenic assay will be completed and the assay validated for possible routine use.

There is considerable interest in the possible differences in immunogenicity between plasma-derived and rFVIII and we intend to compare their immunoreactivity using a variety of methods including immunoblotting, development of recombinant (phage) antibodies (with Dr Ouwehand) and biospecific interaction analysis. In addition, samples of inhibitor plasmas from patients treated solely with rFVIII or pdFVIII will be studied for differences in antibody specificity.

Further basic studies of FVIII, activated FIX, and other ligands are also planned. This is an area in which little has been published with reference to how FVIII activity is related to its biospecific interactions with other proteins and the phospholipid surface: the determination of binding sites and quantitation of binding constants will increase our understanding of FVIII function.

Fundamental structural studies of FVIII may be pursued in a joint project currently under discussion with the Haemostasis Research Group at Northwick Park. Since FVIII has a distinct repeating domain structure (A1-A2-B-A3-C1-C2), if a single domain were produced in large enough amounts for crystallisation (or NMR if a small enough domain), the resulting data would then be immediately applicable by homology modelling to the other similar domains. In a recent report, domain A2 (about 40 kDa) was expressed in a soluble form using a baculovirus expression system. The aim of the project would to increase the yield of A2 as much as possible, purify it and subject it to structural analysis. In addition, a supply of the isolated fragment would be the starting point for ligand binding studies carried out by biospecific interaction analysis.

2. ANTITHROMBOTIC DRUGS

INTRODUCTION

The general strategy in this area has been to develop appropriate methodology for control and standardisation of antithrombotic drugs *in vitro*, and to investigate their mechanisms of action by comparison of *in vitro* measurements with antithrombotic activities *in vivo* using animal models. Ultimately it is hoped that the *in vitro* methods most relevant to the biological activities of these drugs *in vivo* can be defined.

Most of the work has focused on heparin and LMW heparin, which are the most widely used antithrombotic agents. Since the last report 4 LMW heparins have been licensed in the UK and several others are at the pre-licensing stage. Clinical results on a world-wide basis are very encouraging, and in terms of patient numbers this is likely to be the most important drug controlled by the Division.

Work has continued on dermatan sulphate and recombinant hirudin, and studies have been initiated on 2 recombinant proteins currently under investigation as antithrombotic agents - protein C and TFPI.

During the last year a new project has been initiated, funded by the EC, to develop European reference plasmas to assist in laboratory control of oral anticoagulant and heparin therapy. This has already been described in Section 1.1.3 (b).

2.1 CONTROL AND STANDARDISATION OF HEPARIN AND LMW HEPARIN

2.1.1 Control

Unfractionated heparin is a well established product and has been used clinically since the 1940s. Two products, Calciparine (Sanofi Winthrop Ltd) and Heparin Inject (Immuno Ltd) are still under stop orders. Sanofi Winthrop initially submitted protocols only but in view of a potency problem identified with this product, samples from one batch in 6 are now tested. Heparin Inject, the Immuno product, is batch controlled by potency testing (APTT) and protocol examination. Discrepancies in potencies claimed by these manufacturers have been found and 2 batches of the products which had higher than acceptable potencies have been withdrawn. In these cases of potency disagreement, the Division has worked closely with the manufacturers and re-evaluated their house standards which has helped to resolve the discrepancies.

Four low molecular weight heparins: Clexane (Rhône Poulenc Rorer), Fragmin (Kabi Ltd), Logiparin (Novo Nordisk) and Innohep (Leo Laboratories) have now been licensed in the UK. Two of these products (Clexane, Rhône Poulenc Rorer and Fragmin, Kabi Ltd) which were licensed in 1991 are still controlled by batch release. Because batch release of these products is not allowed under the EEC directive Logiparin and Innohep are not subject to batch release and this will presumably be the case for any LMW heparins licensed in the future. However, it is intended to establish agreements with the Medicines Control Agency and

the manufacturers to set up product monitoring systems to check the consistency of these products. In addition, we are committed to continue studies on new LMW heparins at the pre-licensing stage, and products which have already been examined are from Merckle GbM (Germany), Knoll (Germany), Sandoz (Austria), Fidia Srl (Italy), Sanofi Winthrop (France) and Wyeth-Ayerst (USA).

LMW heparin is used clinically as one type of antithrombotic product, but because of its heterogeneity and varying methods of manufacture, each product has its own physico-chemical and biological characteristics. In order to establish the biological profile and consistency of each product, 4 different types of assays (anti-Xa plasma, anti-Xa antithrombin III, anti-IIa antithrombin III and APTT) are performed on each batch. These assays, which were originally performed either on semi-automated coagulometers or microtitre plates, have now been further developed and refined and are routinely carried out on a fully automated instrument (ACL Coagulometer).

The IS for LMW heparin is now used by all manufacturers to calibrate their products. However, despite using the same standard, consistent discrepancies have been found in anti-IIa potencies of Clexane in our laboratory, compared with the manufacturers. This emphasises the need for standardisation of assay methodology, and this situation is expected to be resolved by the manufacturers' adoption of the proposed EP method (see below).

Biological control of these products in our Division is supplemented by physico-chemical studies by Dr Barbara Mulloy in the Laboratory of Molecular Structure. Molecular weight analyses have been performed to check batch consistency and accuracy of stated MWt limits, and NMR studies have helped to define structure/activity relationships and to investigate chemical fingerprints characteristic of certain methods of preparation.

2.1.2 European Standardisation

The wide availability and clinical success of LMW heparin in Europe has accelerated the need for standardisation at the pharmacopoeial level. The EP formed a small working group in 1991 to develop a monograph for LMW heparin and Dr Barrowcliffe was invited to join this group. The main input was assay methodology; both anti-Xa and anti-IIa assay methods for LMW heparin were developed and these methods have been submitted to the EP for inclusion into the draft monograph, which will come into effect in January 1994. Additional input on NMR analysis and MWt determination was obtained by consultation with Dr Mulloy.

More recently LMW heparin has been incorporated into the EP's biological standardisation programme, and Dr Barrowcliffe has been invited by the EP to be project leader. A collaborative study has been organised to investigate the reproducibility of the EP assay methods and to establish a European Standard.

Responsibility for carrying out the collaborative study has been delegated to Dr Gray. The European Standard is intended for use as a house-working standard by the manufacturers of clinical LMW heparins. The collaborative study involves 6 preparations of LMW heparins and 18 laboratories. The study is now in the final stages of analysis and the European Working Standard is expected to be established during 1994.

2.1.3 Standard Independent Units

Based on recent knowledge of the biochemistry of heparin's action, Professor Hemker of Maastricht has proposed a new unit of biological activity which is defined in biochemical terms rather than by standards. One new unit of heparin is defined as the amount of heparin that increases the decay constant of thrombin or FXa by one min^{-1}, in 1 μM of AT III. We are now developing the methods necessary to measure this new unit for unfractionated and LMW heparins, following which we will organise an international collaborative study to investigate the feasibility of using this approach.

2.2 STUDIES OF MECHANISMS OF ACTION OF HEPARIN AND LMW HEPARIN

2.2.1 Antithrombotic Activities of Unfractionated Heparin and LMW Heparins and their Relationship to *in vitro* Anticoagulant Effects

This study was carried out by Dr Gray in collaboration with Dr Barrowcliffe and Dr A Padilla, who was a visiting worker from the Centro Nacional de Farmacobiologia, Madrid, Spain.

One of the major differences between unfractionated heparin and LMW heparins is that LMW heparins all have lower anti-IIa activity than anti-Xa activity; however, the relative importance of these 2 *in vitro* activities for antithrombotic action *in vivo* is still unclear.

In order to elucidate further the relationship between antithrombotic activity and anticoagulant effects of these anticoagulants, we have compared the ability of unfractionated heparin and LMW heparins to prevent thrombus formation in a rabbit venous stasis model, and explored the correlation between their *in vivo* antithrombotic efficacy, their ability to inhibit thrombin generation and their potencies in conventional anticoagulant assays.

2.2.1 (a) **Inhibition of Thrombin Generation: Effects of Platelets on Correlation with Antithrombotic Activity**

It has now been established that when compared with unfractionated heparin, the anticoagulant activity of LMW heparins is more resistant to neutralisation by Platelet Factor 4 (PF_4). Since the anticoagulant activities of heparins are routinely measured in the absence of platelets, this may be one of the major reasons for poor correlation between *in vitro* anticoagulant activity and *in vivo*

antithrombotic efficacy. We have therefore evaluated the ability of unfractionated heparin and 4 LMW heparins to inhibit intrinsic thrombin generation in the presence and absence of PF_4, and of whole platelets, and compared these data with their antithrombotic activities *in vivo*.

The antithrombotic activity of the heparins tested was dose dependent, with unfractionated heparin more effective, on a weight basis, than LMW heparins. Excellent correlation was found between anti-IIa activity and antithrombotic potencies but the correlation between anti-Xa activity and antithrombotic potencies was significantly weaker.

In the thrombin generation test, all 4 LMW heparins and unfractionated heparin inhibited thrombin generation in platelet poor plasma and platelet rich plasma in a concentration dependent manner, with LMW heparins being less active than unfractionated heparin on a weight basis. At least twice as much by weight of each LMW heparin was needed to inhibit thrombin generated in platelet rich plasma than in platelet poor plasma.

As shown in Figure 5 the relative antithrombotic potencies of the LMW heparins were closer to inhibitory activity in thrombin generation with platelet poor plasma than platelet rich plasma. Although the neutralisation of heparins by PF_4 appeared to make a major difference to the actual concentration of unfractionated heparin and LMW heparins required to inhibit *in vitro* thrombin generation, this effect may not contribute greatly to the prevention of venous thrombosis *in vivo* where platelet involvement is thought not to be as high as in arterial thrombosis.

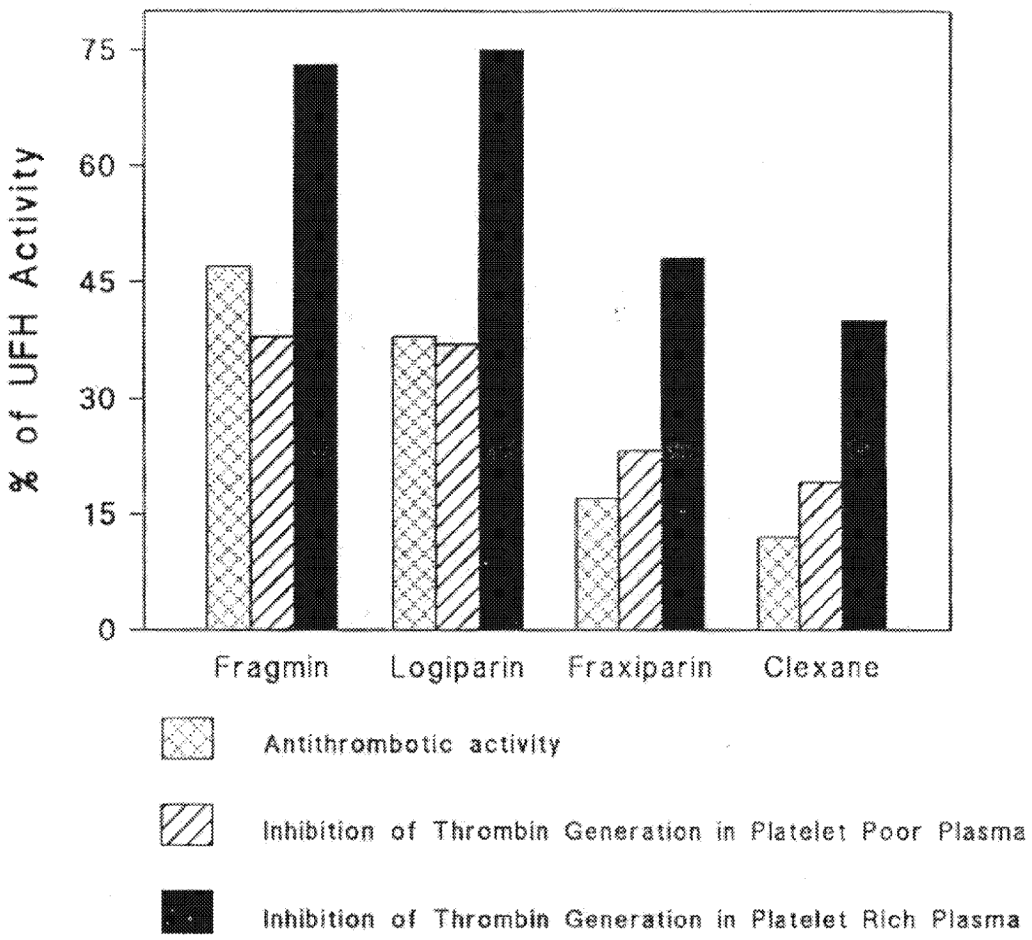
2.2.1 (b) **Inhibition of Intrinsic and Extrinsic Pathways**

Previous studies in experimental models have shown that the antithrombotic potencies of unfractionated heparin and other sulphated polysaccharides differ according to the thrombogenic challenge. We have investigated the ability of unfractionated heparin and LMW heparins to inhibit *in vitro* thrombin generation via the intrinsic and extrinsic systems and compared their relative potencies with their antithrombotic effects in experimental thrombosis initiated by either human serum (intrinsic activation) or tissue factor (extrinsic activation).

In both intrinsic and extrinsic systems *in vitro*, LMW heparins were found to be less potent on a weight basis than unfractionated heparin. Inhibition of extrinsic and intrinsic thrombin generation by unfractionated heparin and LMW heparins were found to correlate better with their anti-IIa activity than with their anti-Xa activity. The results also showed that intrinsic thrombin generation is more resistant to inhibition by heparins, requiring higher concentrations than extrinsic thrombin generation.

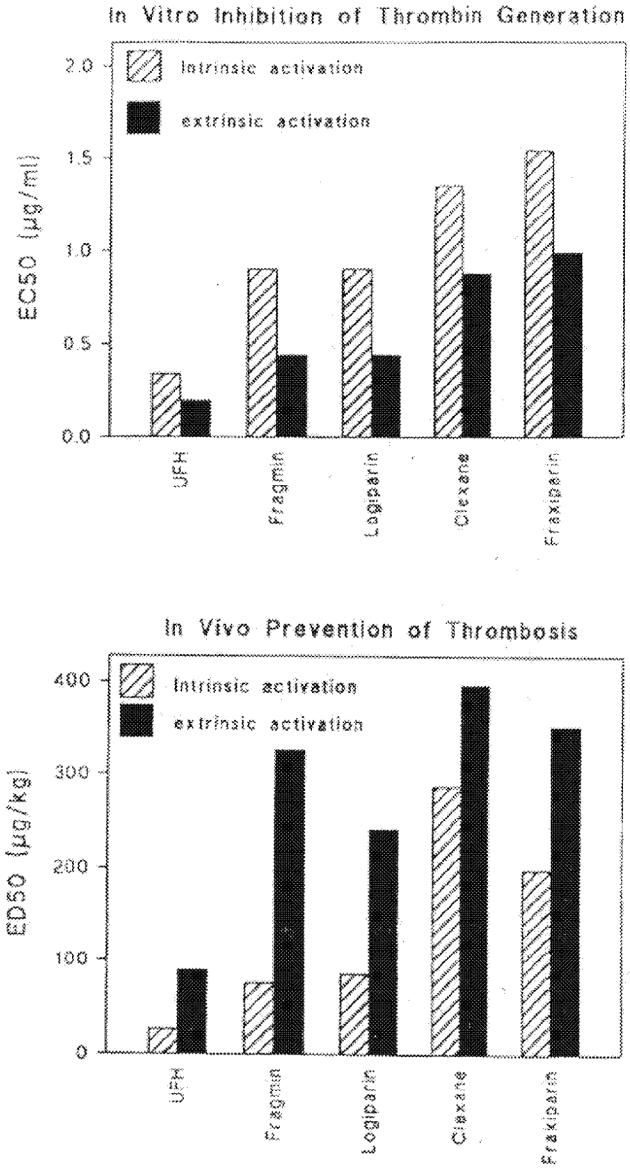
However, these observations were not supported by our antithrombotic studies. As shown in Figure 6 lower concentrations of unfractionated heparin and LMW heparins were required to inhibit thrombus formation induced by human serum

Figure 5 Correlation between antithrombotic activity and inhibition of thrombin generation in the presence and absence of platelets.



Low molecular weight heparins from 4 manufacturers were compared against the 4th IS for unfractionated heparin for their ability to inhibit thrombin generation in platelet rich and platelet poor plasma. When the results of these *in vitro* inhibition of thrombin generation tests were compared with the percentage of antithrombotic activity in an *in vivo* model, the inhibition of thrombin generation in the absence of platelets clearly gave the best correlation.

Figure 6 *In vitro* and *in vivo* activities of unfractionated heparin (UFH) and 4 LMW heparins: comparison of extrinsic and intrinsic systems.



Higher EC50s (concentrations inhibiting 50% of *in vitro* thrombin generating activity) were observed for the intrinsic system (stimulated by glass contact activation, in the presence of phospholipid and $CaCl_2$) than when thrombin generation was triggered via the extrinsic system ie by addition of tissue factor. In contrast, comparing antithrombotic efficacy as depicted by their ED50s (doses of heparins which prevented 50% of thrombosis), both unfractionated and low molecular weight heparins were more effective at preventing thrombosis initiated by stimulation of human serum infusion (intrinsic pathway) than challenges by tissue factor administration (extrinsic pathway).

(intrinsic pathway) than by tissue factor (extrinsic pathway). The disparity between the concentration of heparins required to inhibit intrinsic and extrinsic thrombin generation *in vitro* and the antithrombotic potencies in the serum and tissue factor induced thrombosis cannot be easily explained. It is possible that there is some difference between the human plasma system and a rabbit model or *in vivo* more thrombin is produced via the extrinsic pathway than in the *in vitro* situation. Further investigations are in progress to clarify these observations.

2.2.2 Experimental Studies on a Low Affinity Heparin

This was a collaborative project with Professor D A Lane, Charing Cross and Westminster Medical School. Heparins can be fractionated by their affinity to AT III. Previous studies by Drs Thomas and Barrowcliffe have shown that whilst the high affinity material is responsible for most of the *in vitro* anticoagulant action, the low affinity fraction can potentiate the antithrombotic action of high affinity heparin. In this study, we have investigated the antithrombotic activites and haemorrhagic effects of a low affinity heparin. Compared with unfractionated heparin and dermatan sulphate, low affinity heparin was approximately 6 times less effective as an antithrombotic agent than unfractionated heparin but 5 times more potent than dermatan sulphate. *Ex-vivo* plasma activities suggest low affinity heparin may act by activation of heparin cofactor II and release of TFPI Bleeding time experiments indicate that low affinity heparin presents a significantly lower bleeding risk than unfactionated heparin but slightly higher than dermatan sulphate. We concluded that low affinity heparin is a less effective antithrombotic agent than unfractionated heparin on a weight basis, but it carries a potentially lower haemorrhagic risk. Although low affinity heparin may have a higher bleeding effect than dermatan sulphate, much lower concentration is required to achieve full antithrombotic effect. Clinically, this could be of some importance as the higher concentration and volume of dermatan sulphate required for its successful use is a potential problem with the use of this agent (see Section 2.3).

2.2.3 Effects of Heparin and LMW Heparin on Thrombin Activation of FVIII

Recent studies by the groups of Professor Hemker and Professor Ofosu have emphasised the importance of inhibition of thrombin feedback loops, ie thrombin activation of FVIII and FV, for the overall anticoagulant action of heparins. This study, which was ongoing at the time of the last SPAC report, has since been completed. Unfractionated heparin and 4 LMW heparins were tested for their abilities to inhibit thrombin activation of FVIII in 3 systems. The results showed that at concentrations of each agent sufficient to abolish completely the generation of thrombin in plasma, only partial inhibition of FVIII activation was achieved - complete inhibition required much higher concentrations. It was concluded that other mechanisms as well as inhibition of thrombin activation of FVIII must contribute to the overall anticoagulant actions of heparin and LMW heparin in plasma.

2.2.4 The Effects of Endothelial Cells on the Anticoagulant Activities of Unfractionated Heparin and LMW Heparin

In vivo, perturbations of the vascular endothelium play a major part in the pathogenesis of thrombosis. However, *in vitro* studies on antithrombotic drugs and their mechanism of action are not normally carried out in the presence of the endothelium; this may be the reason why it is often difficult to obtain good correlation between *in vivo* and *in vitro* actions of these agents. The aim of this project is to study the effects of endothelial cells on *in vitro* coagulation tests and to investigate the correlation of antithrombotic efficacy and anticoagulant action in the presence of endothelial cells.

The facilities for harvesting and culturing of endothelial cells from human umbilical cords have been established and apart from this project, endothelial cells are also used for other areas of work. For this project, the endothelial cells are transferred from a primary culture onto a 96-well microtitre plate. Anti-Xa and anti-IIa assays of unfractionated and LMW heparin are then carried out on these microtitre plates in the presence and absence of cells. In the anti-Xa assay, using purified At III, the anticoagulant activity of unfractionated heparin was reduced by 40% in the presence of endothelial cells. For the LMW heparins, reduction in activity ranged from 20-80%. Further experiments are now in progress to investigate the effect of endothelial cells on other coagulation tests. These data demonstrate that endothelial cells can influence the potency of heparins and this effect may account for some of the discrepancy between *in vitro* and *in vivo* activity of these agents.

2.3 DERMATAN SULPHATE

At the time of the last SPAC report dermatan sulphate was under development by industry as an antithrombotic agent following promising results in animals. Work had been initiated on its *in vitro* and *in vivo* biological effects and a potential reference preparation had been ampouled. This work has been extended during the current review period. However the initial clinical studies have been disappointing, because of insufficiently high concentrations in the blood. Work in this area has therefore been suspended pending the outcome of further clinical studies.

2.3.1 Antithrombotic, Anticoagulant and Haemorrhagic Properties of Dermatan Sulphate

Dermatan sulphate is a natural glycosaminoglycan found on the endothelium. It is less sulphated than heparin and it only potentiates the inhibition of thrombin by heparin cofactor II. Dermatan sulphate is known to be an effective antithrombotic agent in animal models, however, a wide range of antithrombotic doses have been reported. In our study, using a hog mucosal preparation, we have shown that dermatan sulphate is much less effective on a weight basis than unfractionated heparin requiring >15 times the dose for equivalent antithrombotic activity. In the rabbit ear Simplate bleeding time model, 2.5

mg/kg dermatan sulphate did not change the bleeding time of the control, while the same dosage of heparin doubled the bleeding time ratio. These studies suggest dermatan sulphate can be used at higher doses as an antithrombotic agent without the increased risk of haemorrhagic side effects. However, there may be difficulties in preparing a formulation with such high concentration of dermatan sulphate for prophylaxis of thrombosis by subcutaneous or intramuscular route.

2.3.2 Potentiation of the Antithrombotic Action of Dermatan Sulphate by Small Amounts of Heparin

We have also investigated the possibility of reducing the dose of dermatan sulphate by adding small amounts of heparin to the preparation. *In vitro* thrombin generation tests of dermatan/heparin mixtures showed that heparin in low concentrations significantly enhanced the ability of dermatan sulphate to impair thrombin generation *in vitro*. These results reflect the *in vivo* efficacy of dermatan/heparin mixtures and we have found that addition of unfractionated heparin at concentrations that do not pose a haemorrhagic risk significantly enhances the action of dermatan sulphate.

2.3.3 Bioavailability of LMW dermatan sulphate

In collaboration with Professor D A Lane, another possibility of reducing the dosage of dermatan sulphate was investigated. Since the bioavailability of LMW heparin is much higher than unfractionated heparin, it was thought that low molecular weight fractions of dermatan may have the same advantage over the parent compound. We have carried out pharmacokinetic experiments on rabbits and found that the bioavailability of the LMW dermatan sulphate was the same as the unfractionated material. Unlike LMW heparin, LMW dermatan sulphate is unlikely to be a successful antithrombotic agent.

2.4 HEPARINOIDS

Heparinoids are defined as sulphated polysaccharides having some structural resemblance to heparin, and since they lack the specific AT III binding site they are usually much less potent as anticoagulants than heparin. Although a number of these products have been available in Europe for several years, none has been licensed in the UK with the exception of a compound from Organon, Oss, The Netherlands.

Organon 10172 (Lomoparan) is a mixture of heparan sulphate, dermatan sulphate and chondroitin sulphate. This has now been licensed in the UK and The Netherlands as an anticoagulant with the main indication for prevention of venous thrombosis. In order to set up assay systems suitable for control and standardisation, we have investigated in detail its *in vitro* anticoagulant and *in vivo* antithrombotic effects using the test systems established for LMW heparin. Although our involvement with the pre-licensing process at the MCA was minimal, and the products cannot be subject to batch release under the EC

directive, Organon has voluntarily submitted the first 6 batches on the UK market to NIBSC for examination.

Previous studies have suggested that unfractionated or LMW heparin may be inappropriate standards for this material and we may have to consider making a separate standard.

2.5 RECOMBINANT HIRUDIN

Hirudin is a small protein (M, 7,000) from the leech which is a specific inhibitor of thrombin. It has been studied for many years and recent work on the kinetics of inhibition, NMR structure and crystal structure when complexed with thrombin have provided a very detailed explanation of its mode of action. Inhibition is reversible and very tight (K_i down to the fM range), with a unique mechanism. The advent of recombinant DNA technology has provided a means of producing large quantities of hirudin for pharmacological use as an anticoagulant. Consequently, 4 European manufacturers are developing recombinant hirudin for clinical use. The previous work on hirudin has been extended in 2 areas; studies of its biological activity in various *in vitro* and *in vivo* systems, carried out by Dr Gray, and standardisation of potency measurements, carried out by Drs Gaffney and Longstaff.

2.5.1 Anticoagulant and Antithrombotic Effects of Recombinant Hirudin

Studies have been carried out, firstly, on the effect *in vitro* of hirudin on various global coagulation tests; secondly, its ability to inhibit thrombin generation and thrombin activation of FVIII *in vitro*; thirdly, *in vivo* its antithrombotic and haemorrhagic effects in rabbits. The *in vitro* results suggest hirudin is a more potent anticoagulant than heparin and 2 other specific thrombin inhibitors tested and hence may be a better antithrombotic agent. However, the APTT data also suggest a strong bleeding risk if the circulating concentrations are excessive. This increase in haemorrhagic risk was confirmed by the bleeding time ratio measured in a rabbit ear template model. At the antithrombotic dose, the mean bleeding time ratio was not prolonged over the control values, but at higher doses, the ratios were higher than those observed for the same dosage of heparin. In the thrombin generation test, higher concentrations of hirudin than unfractionated heparin were required to suppress thrombin generation in human and rabbit plasma, but hirudin was more efficient at inhibiting thrombin activation of FVIII. In experimental venous thrombosis, hirudin was as effective as heparin on a weight basis in the impairment of thrombogenesis. This study adds support to the hypothesis that thrombin inhibition is an important mechanism for antithrombotic activity.

2.5.2 Standardisation of Potency Measurement

Despite our detailed physico-chemical knowledge of hirudin, the literature still contains a number of different approaches for assaying inhibitor activity and a range of specific activities for the purified protein from $<10\,000$ to $>20\,000$

antithrombin units/mg has been reported.

During the last 3 years Dr Gaffney was Chairman of the Thrombin and its Inhibitors Sub-Committee of the SSC of ISTH. During this time a strategy was developed for the control of hirudin and other thrombin inhibitors, which had general acceptance by the international scientific community. The strategy involved the establishment of a highly purified α-thrombin standard for use in assays of these thrombin inhibitors. Following a major international collaborative study an IS for human α-thrombin was established by WHO. In collaboration with Dr Gaffney, and Dr M Y Wong, Informatics, Dr Longstaff designed a protocol to compare recombinant hirudins from 4 manufacturers and a natural leech hirudin in a simple antithrombin assay system, using the α-thrombin standard. The aim of the study was to see if this simple assay procedure would give comparable results in all laboratories (13 participated in the study) for all the hirudin samples (which varied slightly) and whether a hirudin standard (or standards) would be needed. The study was carried out in the first half of 1991 and preliminary results were presented at the XIIIth International Congress on Thrombosis and Haemostatis, Amsterdam, June 1991. The report was well received and a full account of the study was approved by all the participants and is now published. Our findings were that the simple assay procedure worked well in all laboratories with 5 hirudins yielding very similar specific activities between 14 300 and 15 900 antithrombin units/mg.

Although the use of any one of the hirudins as an internal standard gave a further slight improvement in precision, the agreement between laboratories was sufficiently good to obviate the need for a hirudin standard. However, since the stability of the α-thrombin standard is critical it was decided to ampoule and store a reference reagent (4000 ampoules) for recombinant hirudin. The latter will be used to check the storage behaviour of the α-thrombin standard since hirudin is a very stable molecule.

2.5.3 Hirudin Derivatives and other Thrombin Inhibitors

Hirulog and hirugen are 2 recombinant truncated derivatives of hirudin which have been prepared by site-directed mutagenesis; hirulog is in phase II clinical trials, while hirugen is still at the research and development phase. In addition there are a number of unrelated peptides which have been known for some years to have potent thrombin inhibiting activity, though their toxicity remains a barrier to clinical development.

The strategy developed by Dr Gaffney with the use of the α-thrombin standard, and the methodology established by Dr Longstaff, put us in a strong position for standardisation and control of these agents should any of them come into clinical use.

2.6 CYTOKINES, THROMBOSIS AND HAEMOSTASIS

This area of work was initially a joint project with Dr S Poole (Endocrinology). He was responsible for the assays of cytokines while Dr Gray was dealing with the coagulation side. From 1991, Dr Gray has taken the project over completely.

2.6.1 Endotoxin-induced DIC

Bacterial endotoxin has many biological activities and among these is the induction of disseminated intravascular coagulation (DIC). Many of the effects of endotoxin are believed to be mediated by cytokines which are known to initiate the production of the procoagulant tissue factor in certain types of cells. Dr Gray has studied the effects of endotoxin and interleukin-1 (IL-1) on the production of tissue factor and the secretion of interleukin-6 (IL-6) by human peripheral blood monocytes. The effects of the non-steroidal anti-inflammatory drug dexamethasone and antisera to IL-1 on the responses to endotoxin and IL-1 were also studied. IL-1α and IL-1β but not tumour necrosis factor α and IL-6 mimicked endotoxin evoked production of tissue factor and IL-6, suggesting that IL-1 in part mediates disseminated intravascular coagulation caused by endotoxin. Dexamethasone inhibited production of a substance, possibly interleukin-10 or interleukin-8 that limits the tissue factor response to IL-1. Results from experiments with anti-interleukin-1 sera were equivocal. Further work is now in progress to explore the mechanism of action.

2.6.2 Effects of Antithrombotic Drugs on Cytokine Release and Procoagulant Response

Related to this project, an area of work has been initiated to investigate the influence of different antithrombotic agents such as AT III, protein C, activated protein C, TFPI, thrombomodulin and annexin V, on cytokine and coagulation responses to endotoxin. To date, we have studied AT III which is a natural protease inhibitor and has been used successfully for the treatment of disseminated intravascular coagulation induced by sepsis. Preliminary experiments investigating the effects of AT III on endotoxin stimulated production of tissue factor and IL-6 by monocytes have shown that AT III inhibited tissue factor functional activity and secretion of IL-6, but the potency of antithrombin III was dependent on its source; AT III concentrates from different manufacturers showed different degrees of inhibition at similar levels of antithrombin activity. Further experiments to investigate the differences in these concentrates are now in progress.

2.7 THE INFLUENCE OF HEPARIN AND OTHER GLYCOSAMINOGLYCANS ON THE ACTIONS OF GROWTH FACTOR

This project is carried out by Dr Gray in collaboration with Dr C J Robinson (Endocrinology), Dr B Mulloy (Laboratory for Molecular Structure) and Dr E Tarelli (Chemistry).

Heparin and other glycosaminoglycans can modulate the actions of polypeptide growth factors. These effects may help to elucidate the mechanisms of action of the growth factors and are of concern in the clinical administration of heparin, growth factors or combined formulations. The aim of this study is to investigate the effects of different heparins and structurally related compounds on the action of fibroblast growth factors (FGFs). We have examined unfractionated heparin, a series of commercial LMW heparins and other polysulphated polysaccharides such as pentosan polysulphate and dermatan sulphate. These agents were tested for their ability to potentiate the stimulation by FGF of DNA synthesis by fibroblasts and for anticoagulant activity. They have also been characterised structurally by ^{1}H NMR spectroscopy. Our results show that the potentiation of FGF is not related to the anticoagulant activity of the sulphated polysaccharides and that some molecules show higher potentiation of DNA synthesis by acidic FGF, but possess lower anticoagulant activity than unfractionated heparin. The LMW heparins and pentosan polysulphate showed higher potentiation than unfractionated heparin, while the high molecular weight dermatan sulphate has less effect. There was also some correlation between activity and the degree of sulphation, with the highly sulphated compounds more active than the less sulphated molecules. Further experiments are now in progress to confirm these findings and in addition we are investigating the effects of these glycosaminoglycans and growth factors on endothelial cells.

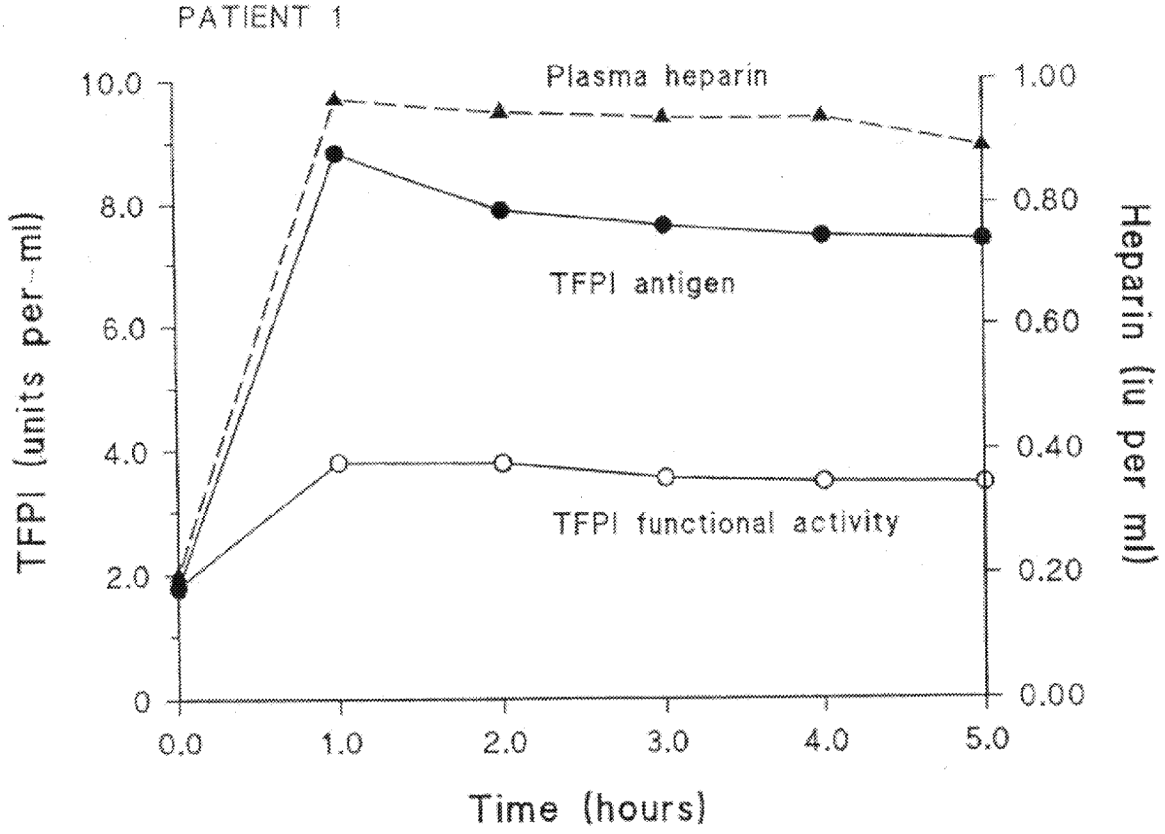
2.8 NOVEL ANTITHROMBOTIC AGENTS

Two naturally occurring plasma proteins, protein C and TFPI, are under investigation as antithrombotic drugs, and in both cases measurements are also made in plasma. Plasma protein C measurements are important for diagnosis of hereditary deficiency states, and an International Standard was established by the Division in 1987. Measurement of plasma levels of protein S, the co-factor for protein C, is also important for diagnosis of hereditary thrombotic tendency, and work has been initiated on standardisation. Assays for TFPI in plasma are still at the investigational stage and their clinical relevance is as yet unclear.

2.8.1 Protein C Concentrates

Products from both plasma and recombinant sources are currently undergoing clinical trials in the treatment of severe protein C deficiency, in the prevention of warfarin induced skin necrosis and in the treatment of sepsis. Concentrates of both the zymogen and activated forms of protein C are under manufacture and samples have been obtained for ampouling. Assays for both antigen and functional activity are in place, and a preliminary collaborative study is being planned to compare the assay of these concentrates against the 1st IS Protein C, plasma. The need for a standard consisting of protein C concentrate will be decided from the results of this study.

Figure 7 Tissue factor pathway inhibitor (TFPI) levels in plasma from a patient receiving intravenous heparin.



The patient received an initial bolus of 3,000 units of unfractionated heparin followed by continuous infusion of 1,000 units per hour.

TFPI functional activity and antigen were measured using the amidolytic and competitive ELISA techniques respectively.

2.8.2 Tissue Factor Pathway Inhibitor (TFPI)

The ability of serum to "neutralise" the procoagulant activity of tissue factor has been recognised for over 70 years. It is only recently, however, that the plasma protein responsible for this effect, TFPI, has been identified and the mechanism of its action understood. TFPI inhibits the procoagulant activity of the tissue factor-factor VII complex, but only in the presence of FXa. It has been reported that the plasma concentration of TFPI increases after heparin administration and this has led to speculation over the possible involvement of TFPI in the anticoagulant action of heparin. As part of an ongoing study in this area we have developed an assay method for the detection of TFPI antigen to be used alongside our existing assay method for TFPI activity.

Earlier attempts to establish a typical "sandwich" ELISA were associated with poor sensitivity and it was found necessary to adopt a method based on the competition between plasma TFPI and recombinant TFPI in binding to rabbit anti-human TFPI IgG. Assays on citrated, normal plasma samples ($n = 16$), by functional and antigen methods gave a highly significant correlation between the results from the two assay methods ($r = 0.841$; $p < 0.001$).

Assays on plasma samples from patients receiving intravenous unfractionated heparin (initial bolus, followed by continuous infusion) showed an increase over the pre-infusion TFPI levels by both assay methods. However, as shown in Figure 7 the increase in TFPI, as measured by the ELISA, was much greater (up to 3-fold) than that detected by the functional assay. Possible interference, by heparin, in the functional assay was prevented by the inclusion of protamine sulphate in the dilution buffer. The reason for the discrepancy between the 2 assay methods in determining TFPI levels in the post-heparin plasma samples is still unclear. However, it is probably related to the different forms of TFPI in normal and post-heparin plasma, eg normal plasma contains mainly lipoprotein-bound TFPI whereas post-heparin plasma contains a large proportion of "free" TFPI. It is possible that the lipoproteins may reduce the binding of the anti-TFPI IgG and therefore we would see an artefactually high antigen value when "free" TFPI was measured relative to normal plasma (lipoprotein-bound TFPI). This conclusion has been supported by assays on full-chain length recombinant TFPI and lipoprotein-free plasma which also showed an increased level of TFPI by ELISA compared to functional assay methods, relative to a normal plasma pool. These results have implications in the interpretation of the effect of heparin infusion on TFPI levels and in the choice of proposed reference materials for the quantitation of TFPI.

2.8.3 Protein S

Protein S is a cofactor for the anticoagulant function of protein C and a deficiency of protein S predisposes to recurrent venous thrombosis. Protein S occurs in two forms in plasma - either bound to the C4b binding protein or free; only the free protein S functions as a cofactor for protein C. Measurement of free protein S requires the prior removal of the "bound" protein S by

precipitation with polyethylene glycol (PEG).

Calibration of the BS Blood Coagulation Factors, plasma, for protein S antigen was prompted by numerous requests for such a standard. In the absence of a primary international standard for protein S it was decided to calibrate the BS by assay against local frozen plasma pools as an interim measure. Most laboratories at the time only carried out measurements of protein S antigen; the specificity of the functional protein S assays is still a matter of debate. Eleven laboratories took part in the calibration exercise for both total and free protein S antigen. Estimates of total protein S were associated with low interlaboratory variability (gcv 6%) whereas estimates of free protein S showed large variability (gcv >20%) when expressed relative to total protein S (eg PEG-precipitated plasma vs whole plasma). However, the variability of free protein S estimates was reduced (gcv 14%) when expressed relative to free protein S in the PEG-precipitated local pools. The results from this study indicate that the largest source of variability is introduced with the PEG precipitation step and that measurements of free protein S antigen in a test plasma should be expressed relative to free protein S in the PEG-precipitated standard plasma.

2.9 FUTURE DEVELOPMENTS

Control and Standardisation

LMW Heparin

The number of batches of LMW heparin submitted under existing licences will probably continue to increase in the short term, and assuming that product monitoring arrangements are established for present and future products not subject to batch release, the control of LMW heparin and related products such as Lomoparan and hirudin could increase substantially. In the longer term, with the adoption by manufacturers of the new EP assay methods, it may be that control work can be reduced, but this will depend on the interlaboratory performance of the new methods.

Following completion of the EP collaborative study on LMW heparins, the main area for investigation of standardisation will be studies of the "standard-independent-units" proposed by Professor Hemker.

Protein S

An international collaborative study with the objective of calibrating an IS for Protein S in plasma is about to be launched. A candidate preparation of normal, pooled plasma has been ampouled for this purpose and it is planned to also include a freeze-dried sample of protein S-deficient plasma in the collaborative assays. The calibration of protein S antigen (total and free) should not pose major problems provided that a few minor instructions regarding assay conditions are specified. However, calibration of the proposed standard for protein S functional activity may be more of a problem since the specificity of the functional assays has been questioned with the recent discovery of a new

activated protein C (APC) cofactor activity. The inclusion of a freeze-dried plasma, deficient in the APC cofactor, but with normal protein S would give valuable information on the behaviour of the different functional assays for protein S.

Tissue Factor Pathway Inhibitor (TFPI)

An interlaboratory study on the assay of TFPI in several different samples (normal plasma, post-heparin plasma, recombinant TFPI) is planned for early 1994.

Development of Techniques

As the number and diversity of antithrombotic agents continues to increase, it is important to establish a variety of techniques which can be used to assess their biological activities. Although ultimately it is intended that mainly *in vitro* methodology should be used, studies of experimental thrombosis in animal models are a valuable adjunct, particularly for new agents, to help evaluate the most relevant *in vitro* parameters to measure.

The Wessler stasis model used up to now has been very useful, but for new agents such as hirudin and antiplatelet drugs it is intended to set up an arterial thrombosis model which may be more relevant to their clinical use. A fibrin accretion model which was used by Dr Gray during her post-doctoral period in Canada will also be established; this is more relevant to the use of antithrombotic drugs to treat established thrombosis.

To complement the *in vivo* study of antithrombotic agents, *in vitro* biochemical studies to differentiate these effects on different parts of the coagulation pathway will be continued. We have already studied in detail the effect on the inhibition of thrombin activation of FVIII; similar systems will be set up to investigate the influence on the inhibition of thrombin activation of FV. It will be interesting also to investigate the effects of these agents on the extrinsic system.

In order to develop more physiologically based *in vitro* methods, the work on effects of platelets and endothelial cells on *in vitro* anticoagulant activities will be extended to cover a wider variety of assay methods and antithrombotic agents.

Research Projects

Antithrombotic Activities of Unfractionated Heparin and Low Molecular Weight Heparins and their Relationship to *in vitro* Anticoagulant Effects

Using tissue factor (extrinsic pathway) as the thrombogenic stimulus, it is intended to complete the present study on the antithrombotic efficacy of the 4 commercial low molecular weight heparins which have already been

investigated extensively in the human serum (intrinsic pathway) system. Studies will be made of the *in vitro* effects of platelets or phospholipids on the inhibition of tissue factor induced thrombin generation by the various heparins and the correlation between these results and the *in vivo* data investigated.

Cytokines, Thrombosis and Haemostasis

Investigations will continue on the interaction of the cytokine network and the coagulation pathway. Dr Poole has agreed to supply the reagents for the IL-1 and IL-6 assays which are already established in Dr Gray's laboratory, while Dr A Mire-Sluis will advise and provide reagents for the measurement of other cytokines (interleukin-4, interleukin-10). The effects of plasma anticoagulants such as AT III, Activated Protein C, TFPI, thrombomodulin and annexin V on cytokine production/release and coagulation by stimulated (LPS, IL-1, tumour necrosis factor) monocytes, lymphocytes and endothelial cell will be studied. In addition, it is proposed to explore, in collaboration with Dr Hockley, an electron-microscopical approach to studies of the inter-relationship between cytokines, endothelial damage and activation of the coagulation system.

As this is a large area of work of fundamental as well as practical interest, it is intended to apply for external funding.

TFPI

Further studies are planned to investigate the contribution of TFPI to the anticoagulant function of heparin; into the effect of lipoprotein association on TFPI assay; the antithrombotic and anticoagulant properties (anti-Xa, anti-tissue factor-FVIIa activity) of purified and recombinant TFPI in the presence and absence of heparin.

3. THROMBOLYTIC DRUGS

INTRODUCTION

The use of thrombolytic agents in the treatment of thrombosis, and in particular in acute myocardial infarction (MI) has continued to increase. These are potentially life-saving drugs and the development and performance of the newer agents such as tissue plasminogen activator (tPA) and Eminase is the subject of intense commercial and clinical interest. tPA was the first synthetic thrombolytic agent, manufactured by recombinant technology and was expected to give improved results over the existing drugs by virtue of its fibrin specificity. However, its performance in several large clinical trials was disappointing, the most recent "GUSTO" trial with 41 000 patients giving only a 1% reduction in mortality for tPA compared to the older drug streptokinase. Manufacturers have therefore been investigating molecular alterations of tPA designed to give a therapeutic advantage, eg prolongation of half-life. A number of these agents produced by recombinant technology eg modified tPA and pro-urokinase are expected to be in clinical trials in the next few years and it is important for the Division to have a strategy in place for their control and standardisaton.

The therapeutic agents currently subject to control and standardisation by the Division are: streptokinase, urokinase, Eminase and tPA. The total number of batches submitted has remained fairly constant at around 47 per year. The current assays used for the control of the major thrombolytic products are simple and reliable, but not amenable to automation or the processing of large numbers of samples. Alternatives are being investigated, specifically using microtitre plate based methods. This will facilitate the assays of larger numbers of samples, for instance when investigating experimental parameters affecting assay performance. This becomes increasingly important as the variety of thrombolytic drugs increases and assays have to be tailored to individual products. As in other areas, the Division's aim has been to develop appropriate control procedures and standards for these materials, and to pursue research programmes which enhance our understanding of their mechanism of action and of the fibrinolytic system through which they operate. Since the last SPAC review, the main development has been in studies of the enzymology of the fibrinolytic system and following a suggestion at the last review, in studies of tPA mutants using a molecular biology approach.

3.1 STREPTOKINASE

There is now extensive evidence from large scale clinical trials of the effectiveness of streptokinase as a thrombolytic treatment for myocardial infarction (MI). Since the 1970's its clinical use has steadily increased and it now represents the "gold standard" against which new treatments are measured. To date no large scale clinical trial has demonstrated that the improvements in mortality rates seen with streptokinase treatment of MI can be substantially bettered by newer treatments. Consequently, streptokinase is the most widely used treatment for MI, in Europe at least, and this is reflected in the numbers of batches submitted for testing where streptokinase outnumbers all other thrombolytics combined. The popularity of streptokinase

may decline in the future as new thrombolytic agents are developed with improved clot specificity which are intended to target fibrin without causing systemic fibrinogenolysis, plasminogenolysis and α_2-antiplasmin depletion, as is the case with all agents in use at the moment.

Control of the 2 products, from Kabi and Hoechst, has not shown any particular problems. However, Kabi have recently applied for a variation to change from a fibrin-based potency assay to a chromogenic assay and following this change we will need to monitor their potencies closely.

3.2 UROKINASE AND LOW MOLECULAR WEIGHT UROKINASE

3.2.1 Background and Enzymology

Pro-urokinase or single chain urokinase type plasminogen activator (SCuPA) is the zymogen precursor of two chain, active, urokinase (uPA). Activation is catalysed by plasmin cleavage of the lys158-ile159 peptide bond. Active uPA is a well established thrombolytic product, more popular in the US and Japan than in Europe. Commercial uPA is isolated from human urine where it is found in 2 forms, native uPA or high molecular weight uPA (HMW uPA, M_r 55 000) and a proteolytically modified form, low molecular weight uPA (LMW uPA, M_r 33 000), which occurs to a variable degree but may predominate. The first International Standard for uPA (code 66/46) also contained this mixture of HMW and LMW enzymes. These 2 forms of urokinase do have somewhat different activity profiles in various plasminogen activation assays, though not in direct chromogenic assays, but this has not been a problem so long as standard and test samples contain similar proportions of HMW and LMW forms. However, with the advent of recombinant DNA technology and high level expression systems capable of producing large quantities of protein, it has become possible to produce purified HMW or LMW uPA and SCuPA. Abbott (Chicago, USA) produce a LMW uPA and are developing recombinant HMW uPA and SCuPA. Two other companies in Europe and one in Japan are also developing recombinant SCuPA to be expressed in either mammalian cell culture or in *E coli*. This situation has led to the development of an International Standard for purified HMW uPA (1st IS for HMW uPA code 87/594), and a preparation of LMW uPA is also available (code 90/642).

The best way to standardise these preparations against one another has not been fully resolved, because their relative potencies vary with the precise assay conditions. We have shown, however, that the differences lessen in fibrinolytic assays with increasing concentrations of substrate (plasminogen). In free solution, activation of plasminogen by HMW uPA may be up to 3 fold more efficient (in terms of k_{cat}/K_m) than LMW uPA. However, using a clot lysis method developed with a microtitre plate format to collect good quality data on replicate samples, the fibrinolytic activities of these enzyme forms can be seen to converge at high plasminogen concentrations in the presence of fibrin.

These results suggest both enzymes have different K_m values but similar k_{cat}

values under these conditions (at saturating [substrate] rate α k_{cat}, but at low [substrate] rate α k_{cat}/K_m). These observations are relevant from a control standpoint since we know from consultations with the manufacturers that Serono assay Ukidan at low [substrate] (plasminogen), Abbott assay their uPAs at very high [substrate], and we routinely use an intermediate [substrate] for control of these products.

3.2.2 Control of Products

The only manufacturer selling urokinase (Ukidan) in the UK at the moment is Serono. Problems have been experienced with inadequate protocols from this company. Occasionally they have been incomprehensible and were returned for corrections, and incorrect information was given on toxicity testing. At the moment Serono are having problems with a new vasoactive substances test, the method now recommended by the EP shows that Ukidan is lethal to rabbits.

3.2.3 Development Work on Prourokinase (SCuPA)

In contrast to uPA, SCuPA is not active in plasma, so when injected or infused does not cause sytemic activation of the fibrinolytic system in the absence of a fibrin matrix. Because of this fibrin specificity, SCuPA is under commercial development as a thrombolytic agent by at least 4 groups. An ideal assay system to standardise doses of this proenzyme might consist of an *in vitro* clot lysis system where SCuPA to uPA conversion could be monitored by plasminogen to plasmin activation and subsequent fibrin degradation, with a feedback loop of plasmin activation of SCuPA. Activity could be compared against a SCuPA standard. However, at this stage it has been decided to adopt a simpler approach of assaying SCuPA in 2 steps, i) activation with plasmin, followed by ii) assay against the HMW uPA standard (code 87/594).

Dr Longstaff has investigated SCuPA activation reactions in detail and shown that the kinetics are not simple. These studies show that SCuPA is able to bind plasmin (and plasminogen) at an allosteric site, causing inhibition of enzyme activity (k_{cat}/K_m is reduced up to 11.9 fold as a result of this interaction). Hence low concentrations of substrate are activated more rapidly. However, other studies show that low concentrations of substrate (SCuPA) lead to incomplete activation whereas high plasmin concentrations produce LMW uPA. In collaboration with 2 other research groups we have now optimised this system and our recommendations on activation conditions were used in a collaborative study carried out in the first half of 1993 on SCuPA standardisation by Dr Gaffney. This follows from a previous study on SCuPA antigen determinations organised in the first half of 1992 to investigate the usefulness of the HMW uPA preparation (87/594) as a standard in antigen assays.

A preliminary report on this study was presented to the Fibrinolysis Sub-Committee at the SCC meeting of the ISTH in Munich, July 1992. It has become apparent from the 1993 study that glycosylated (from mammalian cells) and non-glycosylated (from *E coli*) SCuPAs give different activities in a

fibrin based clot lysis assay, though not in a chromogenic assay. The possibility of different standards for the glycosylated and non-glycosylated products is still under discussion.

3.3. TISSUE PLASMINOGEN ACTIVATOR (tPA)

3.3.1 Control and Standardisation

Only one product, Actilyse (Boehringer Ingelheim) is licensed in the UK and approximately 9 batches/year are submitted. Control of this recombinant product using the IS (87/670) and the clot lysis assay has given no major problems, but minor differences in specific activity between batches were noted by the manufacturers. A small collaborative study was carried out to compare internal standards and the IS, involving NIBSC, FDA, Boehringer Ingelheim and Genentech. This study showed that the internal standards used by Boehringer, Genentech and the FDA were calibrated correctly against the IS.

3.3.2 Carbohydrate Analysis

With the collaboration of the group of Dr Ann Dell (Imperial College, London) we have looked at the carbohydrate sequence of melanoma and recombinant (CHO cell) tissue plasminogen activator (tPA). Differences have been observed. This work was initiated since we observed that melanoma and recombinant tPA's differed in their relative biological activities in 5 different assays. This suggested that discrete (possibly carbohydrate-based or folding-based) differences existed between natural and recombinant molecules. This area is of considerable potential in our future approaches to standardisation. Although no additional work is currently being carried out in this area a watching brief is being kept on the literature, and recently we have negotiated a contract to make a new standard for a non-glycosylated-truncated form of tPA (see following section). Further work in the area of glycosylation and primary sequence mutants will be considered in the future.

3.3.3 Modified tPA

Mutant forms of r-tPA are currently in clinical trial as thrombolytic agents for the treatment of thrombosis. We have demonstrated that the 3 of these studied cannot be calibrated using the current IS for tPA. One of these, a non-glycosylated truncated form of tPA lacking the kringle 1 domain (see Fig 8) may be licensed within the next 2 years. We have found that each mutant form of tPA needs to be separately assessed against the IS for tPA in order to ascertain whether a new standard and a new unitage is required. At the moment we plan to ampoule at NIBSC a separate reference material for the Boehringer Mannheim, recombinant truncated tPA. A distinct unitage will be established for this standard which will essentially be defined after collaborative study of the ampouled preparation.

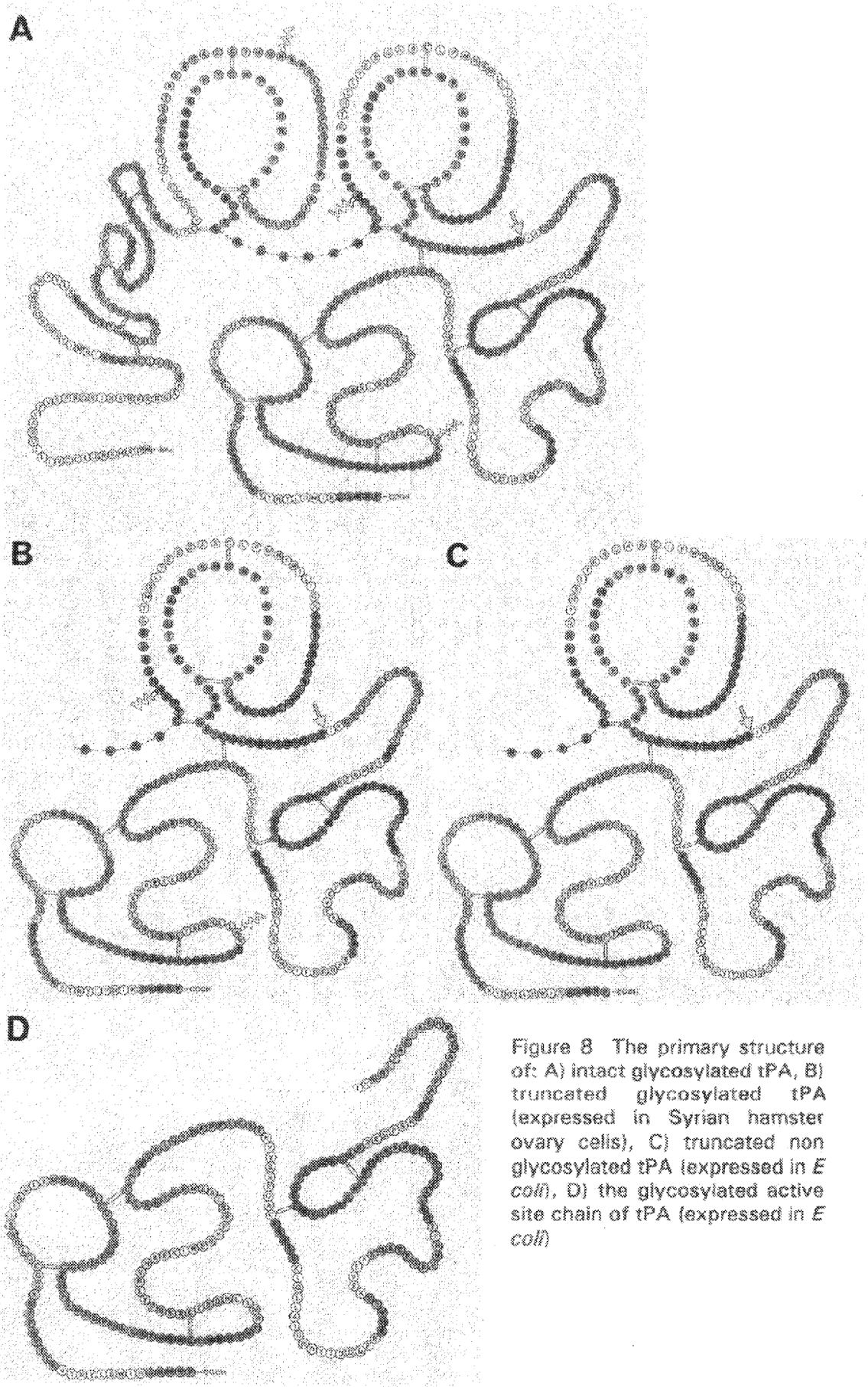


Figure 8 The primary structure of: A) intact glycosylated tPA, B) truncated glycosylated tPA (expressed in Syrian hamster ovary cells), C) truncated non glycosylated tPA (expressed in *E coli*), D) the glycosylated active site chain of tPA (expressed in *E coli*)

3.3.4 Mutagenesis

In 1990, following the previous SPAC review, it was decided that the Division should develop some expertise in the area of recombinant proteins. Dr Longstaff was asked to establish some work involving molecular biology and protein expression because of his previous experience with these techniques. A tPA gene and expression vector for *E coli* were obtained through Dr Gaffney (gene and vector were generously provided by Dr T Cartwright of Rhône-Poulenc), and this formed the basis of 2 research projects carried out on aspects of tPA substrate and inhibitor interactions. The appropriate facilities and equipment for this work had to be built up as none were originally available in the Division. At the present time Dr Valerie Sinniger, a visiting postdoctoral worker from Paris is assisting Ms R E Merton with expression of tPA mutants for activation studies.

One project was to express tPA active site mutants containing catalytic triad to alanine changes. The resulting enzymes would be expected to have intact active sites but have very low proteolytic activity. These enzymes could then be used to study tPA/PAI-1 interactions as a way of discriminating between reversible and irreversible mechanisms of inhibition. According to Dr Longstaff's theories on serpin/protease reactions the inhibitor should still react well with the mutant, inactive enzymes, whereas if cleavage is a part of the inhibitory mechanism, as expected for an irreversible mechanism, PAI-1/mutant tPA binding should be very weak. Mutant tPA genes have now been synthesised and expression work has begun in readiness for binding studies.

The second project involved deletion mutagenesis of tPA, by PCR, to remove approximately half the molecule containing the regulatory domains enabling us to express the serine protease domain only (called tPAsp). This was to be used in activation studies to dissect out the influence of fibrinolytic stimulators (eg fibrin) operating on enzyme (tPA) or on substrate (plasminogen) or both to accelerate reactions. tPAsp has now been expressed, characterised and studied in a variety of activation assays. These studies have provided insights into factors controlling rates of plasminogen activation rates by both tPA and uPA. Results indicate that effectors such as fibrin, fibrinogen fragments, and heparin which stimulate plasminogen activation tend to work through plasminogen conformational changes with uPA as activator, but by a template mechanism with tPA as activator where the template (eg fibrin, fibrinogen fragments, heparin) brings enzyme and inhibitor together to accelerate the reaction. Mathematical models have been developed by Dr C Longstaff to explain these conformational and template mechanisms and these would also be applicable to enzyme/substrate, enzyme inhibitor interactions involving AT/thrombin or FXa, tPA/PAI-1, C1-esterase/C1-inhibitor. Understanding the details of the enzymology of tPA activation will be valuable as tPA deletion mutants approach clinical trials as possible improved thrombolytics. One such protein, called BM 06.022, expressed in *E coli*, developed by Boehringer Mannheim, Germany, is currently being tested in human volunteers with some promising results. As already mentioned (Fig 8) this enzyme lacks approximately half of the regulatory

domain of full length tPA and so consists of the serine protease domain and kringle 2. Consequently it would be expected to have significantly different properties from full length tPA in activation studies.

3.3.5 Single Chain and Two Chain Tissue Plasminogen Activator

Another aspect of tPA action which has been studied recently is the effect of single chain to two chain conversion on specific activity in different assay situations. tPA is an unusual serine protease in the coagulation and fibrinolysis cascades in that it is not produced as a single chain zymogen requiring proteolytic activation, but is released from the endothelium as an active enzyme. However, activity in free solution is low until the enzyme is stimulated by the binding of various promoters, such as fibrin, fibrinogen fragments, heparin etc. This template dependent action is the basis for tPA's clot specific activity. Nevertheless, two chain tPA (tctPA) is formed from single chain tPA (sctPA), by plasmin, but this appears to lead to only small improvements in specific activity.

Dr Longstaff has investigated this question further looking at sc- and tctPA activities with a range of promoter molecules known to stimulate enzyme activity in vitro. Previous work has led to a widely held mechanism where stimulators of activity act by binding sctPA to induce a conformational change to a more "tctPA-like" structure. Hence, activation has been supposed to occur either by tctPA formation or by induction of a tctPA-like conformation in sctPA resulting from promoter binding. Our results suggest this is an oversimplification. Conformational changes may be induced in sctPA by fibrin binding, but not by other promoters, eg fibrinogen fragments or heparin, which work by a template mechanism only. These observations are relevant to tPA assay design since the *in vivo* activity of tPA must involve both template and conformational mechanisms, and *in vitro* assays should be designed to reproduce this. Thus one of the favoured assays for tPA using soluble fibrinogen fragments as promoter (the Verheijen assay) is not recommended. A fibrin-based assay system, as used in the Division here, is preferred since it makes sc- and tctPA look similar, avoiding complications due to tctPA generation during the assay, and requires only one standard for tPA rather than both sc- and tc- varieties. Another interesting observation from these studies has been the generation of a new form of low molecular weight tPA resulting from prolonged plasmin treatment of sctPA, which appears to have better fibrin specificity than sc- or tctPA.

3.4 EMINASE

This is the trade name for acylated plasminogen streptokinase activator complex (APSAC) manufactured by Smith Kline Beecham. It was hoped that the fibrin affinity and slow deacylation *in vivo* of the complex would make it an effective competitor to tPA and streptokinase. This has not materialised and the number of batches of Eminase which we have controlled during the last 4 years has remained quite small. The product is assessed for potency and fibrin binding

using an internal standard established by the manufacturer, part of which is deposited at NIBSC. The unitage of this standard was initially defined by the manufacturer on the basis of clinical data. No problems have been experienced with these assays. The plasminogen for this product is a blood product supplied by Immuno, Austria for which a separate product licence exists, and Immuno submit to NIBSC separate samples of plasminogen and the plasma pools used for its manufacture. Recently one plasma pool was found by NIBSC to be positive for hepatitis C antibody and Smith Kline Beecham withdrew several batches of product in the UK and Europe.

3.5 BASIC ENZYMOLOGY OF FIBRINOLYSIS

Dr Longstaff has used his expertise in enzymology to investigate some of the important molecular interactions in fibrinolysis, especially those between enzymes and inhibitors. The methodology developed can be applied quite broadly in both fibrinolysis and coagulation systems, and as already mentioned has been used for basic studies on AT III. Work on the enzymology of pro-urokinase and tPA has already been described in Sections 3.2 and 3.3.

3.5.1 Plasmin

Plasmin is a key enzyme in fibrinolysis as the serine protease responsible for digesting fibrin, although it has roles in other systems including inflammation, tissue growth and development and possibly tumour metastasis. Plasmin is also the enzyme generated and measured during plasminogen activator assays, so it is essential we understand the enzymology of this protease sufficiently well. In a previous study on the kinetics of SCuPA activation by plasmin it was shown that plasmin (and plasminogen) bind SCuPA through kringle domains and this reduces enzyme activity. This appeared to be a form of negative cooperativity or substrate inhibition. However, it was subsequently discovered that this inhibition of SCuPA activation or indeed of plasmin amidolytic activity on a simple chromogenic peptide substrate could be achieved by a number of unrelated proteins, albumin, IgG, fibrinogen. Thus plasmin activity is regulated by protein concentration, an unusual finding. It is not clear what significance these interactions have *in vivo*, but plasmin appears to be a "sticky" protein, very sensitive to its environment. It is also possible that this phenomenon is more widespread amongst those proteases with large regulatory domains found in fibrinolysis and coagulation. This has important implications for the design of assays and also for preparation of standards both of which often include "inert" proteins such as human serum albumin (eg most of the standards used in fibrinolysis contain albumin as a bulking agent and stabiliser).

More recent work on plasmin has been the development of a reliable experimental system to investigate activity in the presence of fibrin which acts as both substrate and effector. This work is carried out using a microtitre plate-based clot lysis system to generate good quality data from replicate wells. This has already provided some interesting results in kinetic studies using aprotinin and tranexamic acid as inhibitors and illustrating that different mechanisms of

inhibition can operate in free solution or in a fibrin milieu. Assay of plasminogen activators is also being investigated using the microtitre plate format. This will be helpful for routine control work on existing thrombolytic products and also in developing new assays for novel products. At the present time this assay approach is working for streptokinase, urokinase and tPA and is being optimised for routine use to replace the traditional "glass bead" assay.

Dr Longstaff's experience with plasmin has also been called upon by Dr Thorpe in the Division of Immunobiology and by BPL who have been having difficulties with fragmentation of batches of intramuscular IgG, occurring during the shelf life of the finished product. It was found that sensitive batches contain high levels of active plasmin which are generated during production and storage. It was suggested that this may be the result of kallikrein activation of residual plasminogen, since these batches of IgG have also been found to contain very high levels of prekallikrein activator, higher levels than comparable products from other manufacturers. BPL are attempting to modify their production methods to eliminate this problem and we are monitoring the situation.

3.5.2 α_2-antiplasmin

This is the major inhibitor of plasmin in plasma and is one of a class of inhibitors known as serpins (serine protease inhibitors). Several serpins are inhibitors relevant to fibrinolysis and others have roles in the related areas of coagulation and inflammation. Understanding the mechanism of action of an inhibitor is a necessary step in deciding how an inhibitor should be assayed and potency determined, as discussed below. Initial studies were on the interaction of α_2-antiplasmin with plasmin or chymotrypsin as model serpin and serine proteases to investigate binding mechanism. Plasmin is obviously an important enzyme for fibrinolysis, but has a reasonably broad specificity and is implicated in a number of other processes including inflammation, tissue development and repair, and possibly tumour metastasis. The proteolytic activity of plasmin is rapidly quenched in plasma primarily by α_2-antiplasmin, but in the presence of fibrin and some soluble analogues inhibition is much less effective.

Conclusions from these studies were that serpin binding kinetics were the same as other small protein protease inhibitors, suggesting a similar mode of action. Specifically this means that both types of inhibitors are reversible, contradicting the prevailing wisdom at the time which isolated serpins in a class of irreversible inhibitors. In addition, evidence was put forward against the accepted mechanism explaining plasmin resistance to α_2-antiplasmin in the presence of fibrin.

Following on from this a direct comparison was made of inhibition of plasmin by α_2-antiplasmin and a classic small protein inhibitor, aprotinin (also known as Basic/Bovine Pancreatic Trypsin Inhibitor or BPTI and sold commercially as Trasylol, used to reduce post operative bleeding after major surgery). Aprotinin is a member of the Kunitz family of protease inhibitors and is one of the most intensively studied proteins in nature. It is well known to be a reversible

inhibitor of plasmin, trypsin, chymotrypsin, kallikrein and urokinase. The work carried out highlighted the similarities in binding kinetics for plasmin inhibition by the serpin, α_2-antiplasmin and the kunitz inhibitor aprotinin in a direct comparison. This work was presented at a meeting of the Haemostasis Club in 1991 and led to Dr Longstaff being awarded the Porton Silver Medal.

Part of this work addressed the problems of expressing inhibitor binding strength and measuring correctly inhibitor potencies where very high affinity interactions were involved. The strength of a reversible inhibitor is stated as a K_i value (unit M, the same as K_d); whereas with an irreversible inhibitor potency is expressed as a second order rate constant k_{on} (units $M^{-1}s^{-1}$, analogous to k_{cat}/K_m for an enzyme substrate reaction). Choosing an inappropriate unit on the basis of the wrong choice of reversible or irreversible mechanism will provide an incorrect answer for inhibitor potency or even specificity. Theoretical aspects of measuring inhibitor potency have been expanded upon in a subsequent publication. Here it was shown that establishing the correct reaction mechanism is an essential step in designing inhibitor assays and measuring inhibitor strength. This means determining whether binding occurs irreversibly, reversibly, in a simple one step manner or in multiple steps. In fact the distinction between reversible and irreversible binding can have some surprising consequences for inhibition kinetics. For example binding equations show that for a reversible inhibitor it is possible that under certain conditions the slower it interacts with its target enzyme the better an inhibitor it becomes. This is also clear in practice. This behaviour is not widely appreciated, but is clearly important if serpins are reversible inhibitors. Furthermore, the kinetic consequences of a reversible mechanism become more important when mutant proteins (naturally occurring or made by recombinant DNA techniques) are investigated or when inhibitors are used against their non-target enzymes.

3.5.3 C1 Inhibitor

Following publication of some of the work discussed above, a collaboration was set up with a group working at the Central Laboratory of the Dutch Red Cross, Amsterdam, who were working on C1 inhibitor, a plasma serpin with activity against complement proteases (C1r, C1s), coagulation proteases (factors XI_a, XII_a and kallikrein) and plasmin. Kinetic analysis of the binding of C1 inhibitor variants (expressed in mammalian cells) to a variety of enzymes was performed. The collaboration proved successful utilising the same techniques applied to plasmin and α_2-antiplasmin. Reversible interactions were again observed for a range of C1 inhibitor analogues with mutations in the P1 site and P3/P5 sites.

3.6 PLASMINOGEN ACTIVATOR INHIBITOR 1 (PAI-1)

The major inhibitor of tPA and urokinase in plasma is called PAI-1. There is increasing interest in measurements of the PAI-1 level in plasma and other biological fluids. It has been suggested that a high plasma PAI-1 level

predisposes to thrombosis, notably myocardial infarction. Following 2 international collaborative studies of a PAI-1 preparation in plasma the Fibrinolysis Sub-Committee of the SSC of ISTH agreed that the preparation should be calibrated to contain 25 units of tPA neutralising activity and 175 ngs of antigen. Since only a small number of ampoules of this preparation remain we have ampouled a second prospective standard at NIBSC and plan to study this during 1994 with a view to establishment as an international standard. This work is being conducted in collaboration with the Fibrinolysis Sub-Committee of the SSC of ISTH.

3.7 MONOCLONAL ANTIBODIES (MABS) TO FIBRIN

This area of Dr Gaffney's work has been largely supported by 2 external grants: i) from the Wellcome Trust (£60 000, 3 years), in collaboration with Dr P Webbon of the Royal Veterinary College (RVC). This work was carried out by Mr F McEvoy, a lecturer at RVC, largely at the College, and by Ms T Edgell, a scientific officer, largely at NIBSC. The grant terminated in October 1993. ii) from the University of Leuven (£70 000, 3 years).

3.7.1 *In vivo* and *in vitro* Studies

Five fibrin-specific mabs have been studied. Fab^2 and Fab have been isolated for each. Labelling with Technetium has presented F McEvoy with major problems and a suitable procedure for each mab has not yet been established. However, F McEvoy using I^{131} label has confirmed our earlier work of imaging human clots in the jugular vein of the rabbit and in the saphenous vein of the sheep. Work is continuing during the next 3 years (with Dr S Raut) on the identification of the epitopes in fibrin for each of the mabs. Two of the mabs (12B3B10 and IH10) are being 'humanised' in the laboratory of Professor Collen (Leuven, Belgium).

Further work on the mabs has been performed in which 2 of the mabs were identified which crossreact quite avidly with pig and dog fibrins. The binding profile for each mab following plasmin treatment of fibrin was established and explained the enhanced binding of the D dimer mab to fibrin during thrombolytic therapy with antibody-lytic agent. These data also allowed the best choice of mab for subsequent work on cloning a therapeutic mab/lytic agent construct. It is planned to continue this work with the Leuven group. Dr Raut will continue to isolate the $A\alpha$ Bß and γ chains of fibrinogen and analyse various peptide compositions from each chain in order to provide reagents for the study of linear epitopes to which the individual mabs are directed.

3.7.2 Fibrin Aggregation

To compliment our fibrin mab work we have continued studies of fibrin aggregation. Significant progress has been made in developing methodology to understand the stage and order of fibrin binding of many components of plasma which bind to fibrin (α_2 antiplasmin, fibronectin, plasminogen, PAI-1,

thrombin etc). It is planned to mark out selectively various of the components which bind to fibrin and examine the effect both on structure of fibrin and its resultant lysability. We have demonstrated that one only of our 5 fibrin-specific mabs (MB3B10) seems to enhance dramatically the fibre thickness of fibrin. It probably behaves as a divalent bridge between the 2 carboxy terminal ends of 2 distinct fibrin subunits. This remains to be proven by using the already generated Fab fragment of this mab.

3.8 FUTURE DEVELOPMENTS

Control and Standardisation

New products currently under development or in clinical trials include a variety of tPA mutants with improved plasma half-life and enhanced fibrin binding or proteolytic activities; pro-urokinase (SCuPA), which possesses some fibrin specificity by an unknown mechanism; chimeric molecules of tPA fibrin binding domains linked to the uPA serine protease domain which is a more active protease; chimeras of tPA or uPA linked to fibrin specific antibodies; staphylokinase, a molecule with a similar mode of action to streptokinase but possibly with some fibrin specificity. Assays and standards will have to be developed for some of these proteins as they reach the clinical stage of development.

Our approach will have to take account of the production of recombinant molecules differing to varying extents from the native molecule. Each new thrombolytic agent which relates to tPA needs to be individually assessed for comparability with the current tPA standard. It seems that we may be involved with many other therapeutics in the future which have no natural counterpart and are essentially the result of gene manipulation in order to improve the performance of a molecule. Each case will have to be examined separately.

In the area of fibrinolysis many of our standards are highly purified preparations. These have proved quite unsatisfactory to calibrate ELISA assays in plasma, non parallelism being found between the standard and test. Thus we plan to generate a range of plasma standards for such molecules as tPA, uPA, PAI-1, PAI-2, plasminogen etc. This is a high priority and a standard for plasma tPA will be ampouled in 1994.

Research

Enzymology

Results already obtained emphasise the significance of understanding the enzymology of protease activation and inhibition processes for our understanding of haemostasis and fibrinolysis *in vivo*. Sound enzymology is a prerequisite for good assay design for control and standardisation work, if these assays are to reflect realistically *in vivo* potencies. At the present time we believe we have identified most of the important components and the main

interactions of fibrinolysis and coagulation, but in reality we are a long way from a complete understanding of the dynamics of fibrin formation and lysis *in vivo*, so much work remains to be done. Much work has been done on the individual reactions and interactions in these pathways, and over time it is hoped these can be extended and combined into mathematical models and computer simulations of these biochemical pathways. This is a longer term goal, but some modelling work has begun. This will be developed further, for example to investigate the interelationship between coagulation and fibrinolysis during thrombolytic therapy. This area is currently generating much interest, and directly relates to optimising regimes of treatment for MI with best combinations of thrombolytics and anticoagulants. External funding will be sought for this work. These studies will shed light on the operation of these pathways *in vivo*, helping us to design appropriate *in vitro* assay systems to determine good approximations of *in vivo* potencies of enzymes, inhibitors and cofactors.

Templates play a major role in regulating a variety of reactions in coagulation and fibrinolysis. Some examples are fibrin, soluble fibrin(ogen) fragments, heparin, and various cell surface components. Initial work has begun on understanding the effects of template action using mathematical models with tPA and heparin as a simple system. Another potentially very interesting direction is in the area of cell surface components as templates, including protein receptors and glycosaminoglycans. These are known to influence reactions involving tPA, SCuPA, plasmin(ogen), and α_2-antiplasmin. It is hoped that a fruitful collaboration can be established between the Division and Dr Jordi Felez, Head of the Department of Cellular Receptors at the Cancer Research Institute, Barcelona, Spain, to explore these reactions. We have recently been successful in obtaining a grant from the British Council to initiate this collaboration between our 2 laboratories, and we hope to secure further funding to continue this work.

At a molecular level, structure/function studies will continue on tPA using domain deletion to understand the role of the regulatory domains in tPA and point mutagenesis to investigate enzyme inhibitor and substrate interactions. These studies are of interest in view of the intensive programmes underway worldwide to develop "third generation" thrombolytics with improved clinical profiles. Many of these will be variants of current products including tPA. We would like to extend these studies to other relevant proteins including SCuPA and uPA, plasminogen etc. Ideally we would also like to obtain new structures in collaboration with protein crystallographers; this would generate new opportunities to apply protein engineering studies to further our understanding of protein action at the atomic level, and open up many new lines of investigation.

Fibrin and Clot Lysis

It is intended to continue the work on the characterisation of fibrin-specific mabs partly in collaboration with the Leuven group, and further attempts will

be made to integrate the mab work with the fibrin aggregation studies.

Considerable work on the clot lysis assay is planned. Our prourokinase collaborative study has indicated that the form of the clot lysis test influences the comparability of molecules having structural differences but having the same basic mechanism.

4. TRANSFUSION MEDICINE AND GENERAL HAEMATOLOGY

INTRODUCTION

Compared with the area of haemostasis and thrombosis, which has been a major focus of the Division's work since its inception in 1976 the Division's involvement in the field of transfusion medicine is fairly recent, and the resources devoted to this area have been relatively minor. The introduction of legislation on product liability, the need for a single UK voice in Europe on transfusion matters, and the practical need for reference and working standards have all accentuated the need for regulation, standardisation and control of the activities of Blood Transfusion Centres.

In some ways a modern Regional Transfusion Centre (RTC) can be compared to a small pharmaceutical factory, but there are many important differences. Probably the most crucial difference is that each single donor "product" in an RTC, eg red cells, platelets, plasma is effectively unique. This makes quality control difficult, and batch release, such as is performed at NIBSC for other products, virtually impossible. However, there are many other ways in which quality control of products can be improved eg by development and definition of methods, provision of reference materials.

In the last 4 years NIBSC has provided major support to the UK Blood Transfusion Service in 4 areas:

1) Development of Guidelines

Following the successful publication in 1990 of the first UK BTS/NIBSC Guidelines for the Blood Transfusion Service, work continued on revision and refinement via several standing committees, and a second edition was published in 1993. Dr Barrowcliffe chaired the Committee on Plasma for Fractionation and Dr A R Hubbard, Dr G Kemball-Cook and Dr R Thorpe were also members. Dr P Minor was a member of the Committee on Donor Selection and Dr P Phillips was a member of the Committee on Reagents.

2) Research and Development in the Division of Haematology

The work on immunohaematology of anti-D, which had been initiated by Dr Susan Thorpe at the time of the last SPAC review, has been extended and expanded, and will be described in detail in this Section. A new area of work has been started, on platelets, with the focus on immunological aspects, and for this purpose Dr W Ouwehand, a Consultant Haematologist and Senior Lecturer at the Cambridge RTC has been given a part-time appointment within the Division of Haematology with the remit of establishing a programme of work in this area.

3) Blood Group Serology

The National External Quality Assessment Scheme (NEQAS) in blood group serology is run from NIBSC. The scheme manager is Dr P Phillips, Head of Standards Division and the work is physically located in this Division. However, since it forms an

important part of the Institute's programme on Transfusion Medicine, it is being included in this review. In addition, Dr Phillips is also responsible for production of some reference and working reagents in blood group serology.

4) Working Standards for Screening of Donors for Viral Markers

This was identified as a major need in discussions with the Transfusion Service and the first standard, for hepatitis BsAg, has recently been established. This work is done in the Division of Virology and was recently reviewed as part of that Division's programme - it will not be described further here.

In addition to the work on transfusion medicine a small amount of work is done on standardisation in general haematology, and for convenience is described in this section since it is the responsibility of Dr Susan Thorpe.

4.1 IMMUNOHAEMATOLOGY

Much of the Division's work in this area has centered around anti-D. The Rh D antigen is clinically the most important blood group antigen after A and B since D-negative women can become immunised against D if D-positive erythrocytes enter their circulation during parturition; the resulting antibodies can cause potentially fatal haemolytic disease of the newborn (HDN) in any subsequent pregnancies involving a D-positive baby. However, administration of anti-D IgG following parturition suppresses maternal immunisation and since the introduction of anti-D prophylaxis in the late 1960s the incidence of HDN has been dramatically reduced.

4.1.1. Standards

2nd British Working Standard for Anti-D Antibodies

This Standard is intended for use in the assay of plasma anti-D by automated haemagglutination using autoanalysers. The 1st Standard (72/229) was established in 1975, but has recently been replaced by 73/515, derived from the same pool of plasma as 72/229 and included in the original collaborative study. Although 73/515 was assigned the same potency as 72/229 (11.5 iu/ampoule), both being calibrated against the IS for incomplete anti-D blood typing serum, in view of the time that had elapsed since the original study, a limited collaborative study involving 4 Transfusion Centres was undertaken to recheck the potencies of 72/229 and 73/515 against the IS. The statistical analysis was carried out by Mr A Heath of Informatics.

The results showed that preparations 72/229 and 73/515 were still indistinguishable and the mean potencies fell within the range of potency estimates in the original collaborative study. To ensure continuity, 73/515 was therefore assigned a potency of 11.5 iu/ampoule.

IRP for Anti-D Ig

This material (established 1977) is intended for the calibration of local or national standards used in the assay of anti-D immunoglobulin preparations and is mainly issued to manufacturers of these products.

British Working Standard for Anti-c

The success of anti-D prophylaxis has led to a dramatic decrease in the incidence of women becoming immunised against the D antigen. This has meant that immunisation against other antigens, particularly Rh c, has now become clinically more important than previously. The Division therefore distributes a working standard (produced by Dr P Phillips of NIBSC) intended for use in the measurement of anti-c in plasma by automated haemagglutination.

4.1.2 Control-related Activities

Agglutinating Activity in Therapeutic Ig Preparations

An adverse haemolytic reaction in a patient who had received a therapeutic normal immunoglobulin (virtually all IgG) preparation was recently reported to the Division of Immunobiology which controls these products. It was suggested that the adverse reaction could have been caused by the presence of antibodies against the blood group A antigen. This batch of immunoglobulin was therefore tested 'blind' by haemagglutination (indirect anti-human globulin test) along with 10 other batches in order to ascertain whether it contained unusually high levels of IgG anti-A. Due to the high immunoglobulin concentration in these preparations and the low titres of anti-A (and anti-B), the standard indirect anti-globulin methodology had to be modified. The results showed that the titres of IgG anti-A and anti-B in the immunoglobulin preparations were very low (usually 1/5-1/10) and that in this respect the suspect immunoglobulin batch was no different from the other batches tested. The possibility that the adverse reaction suffered by the patient was due to the presence of unusually high levels of anti-A in the immunoglobulin preparation was therefore ruled out.

Development of an In-house Assay for Measuring Anti-D Potency of Prophylactic Preparations

The use of anti-D immunoglobulin for *in vivo* clinical use requires *in vitro* quantitation of anti-D potency. Anti-D potency is usually determined by comparing the quantity necessary to agglutinate D-positive erythrocytes with the quantity of a reference preparation, calibrated in IU, that is needed to produce the same effect. This may be carried out by manual titration (using albumin displacement, enzyme treatment techniques or the indirect antiglobulin method), or, more commonly, by automated haemagglutination using autoanalysers. The latter method is used by Transfusion Centres and is the EP reference method. However, this technique requires specialised equipment and an experienced operator, and is unsuitable for use in general laboratories.

Human monoclonal anti-D antibodies are currently being evaluated for prophylaxis since there is a shortage of polyclonal anti-D. Their clinical use will also necessitate functional quantitation to check that the purification procedure has not impaired the desired biological properties of the antibody and to ensure batch to batch consistency. Prophylactic anti-D immunoglobulin preparations are controlled at NIBSC so it seemed worthwhile to develop an in-house assay of anti-D potency.

Advantage was taken of the availibility of monoclonal anti-D antibodies to develop a specific, radioimmunoassay for anti-D potency in which labelled monoclonal anti-D and unlabelled anti-D compete for antigen ie red cell binding.

Three human monoclonal anti-D antibodies, Fog-1, Brad-3 and Brad-5, kindly provided by the BPL, Elstree, were used as ^{125}I-labelled tracers in the radioimmunoassay.

To determine assay reproducibility, the effect of using different monoclonal anti-D as tracers, and to make a comparison with the autoanalyser method, 4 batches of products licensed for use in the UK (designated A,B,C and D; from 3 manufacturers) were assayed against the IRP for anti-D immunoglobulin. Each sample was assayed up to 6 times with each ^{125}I-labelled monoclonal anti-D tracer in separate assays using at least 2 individual ampoules of the IRP and R_2R_2 cells from at least 2 single donors. Potencies were determined by standard parallel line assay methods.

For each preparation and monoclonal antibody, overall means were calculated as geometric means, and repeatability of assays measured by the geometric coefficient of variation expressed as a percentage of the mean (%gcv). This analysis was carried out by Mr A Heath. The results are shown below.

Anti-D Potencies (iu/ml) Determined by RIA

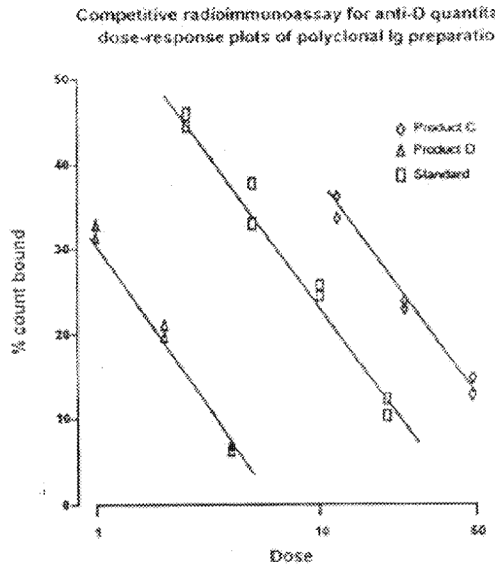
Product	A	B	C	D
Label	146	158	138	1250
Brad-3	142 (3.7)	147 (1.8)	129 (2.6)	1797 (2.9)
Brad-5	143 (4.4)	145 (5.0)	125 (3.8)	1824 (3.0)
Fog-1	130 (7.8)	121 (5.7)	124 (2.3)	1836 (5.6)

The results for Brad-5 and Brad-3 agree closely, and, for products A, B and C, are nearer to the stated potencies determined using autoanalyser methodology than the results using Fog-1. Although all 3 monoclonals gave a higher potency for product D than the stated potency, the difference is well within the variation in potencies amongst different laboratories using autoanalysers.

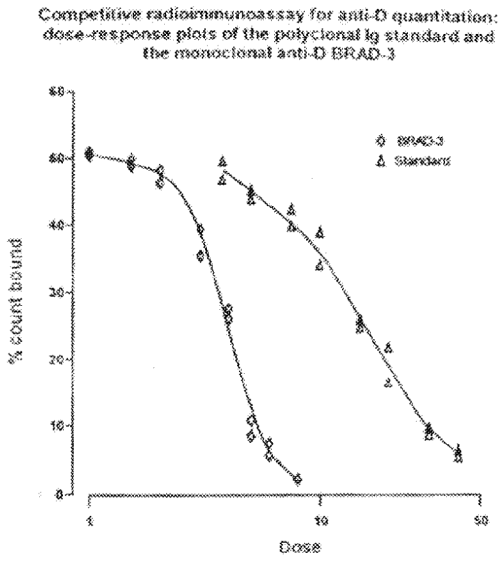
The radioimmunoassay was also used to measure the potencies of Brad-3 and Brad-5 preparations, provided by BPL (using ^{125}I-labelled Brad-3 and Brad-5 respectively), against the IRP.

Figure 9 Quantitation of anti-D immunoglobulin by radioimmunoassay

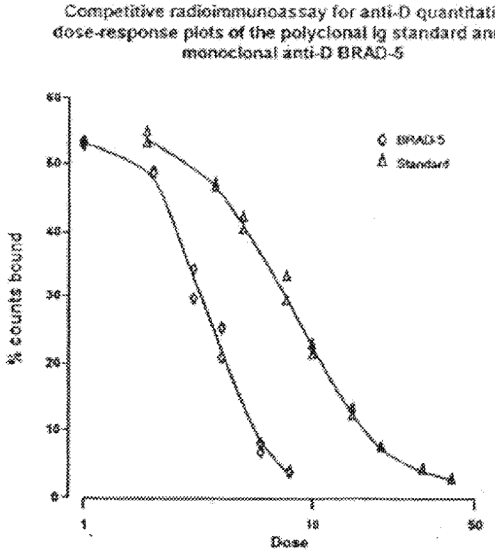
A



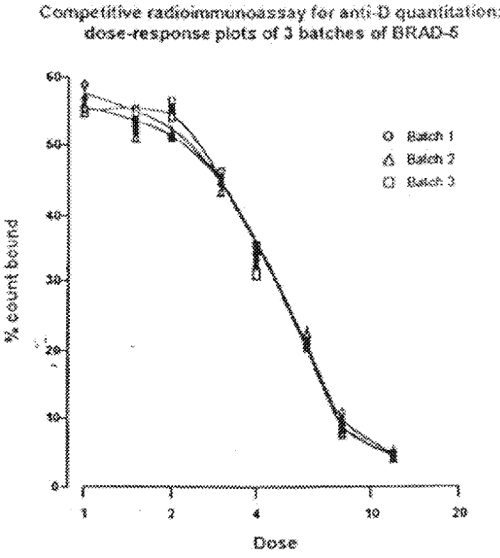
B



C



D



However, as might be expected when assaying a monoclonal antibody against a polyclonal standard, the dose-response curves were not parallel (Fig 9). Nevertheless, using the entire length of the curves, overall potencies were 11.4 iu/ug for Brad-3 and 7.5 iu/ug for Brad-5 (average of 2 assays). These values compare with 5 iu/ug for polyclonal anti-D.

Different batches of Brad-5, all containing the same amount of immunoglobulin protein, were also assayed. In this case, the dose-response curves were not only parallel, but overlying indicating that each batch had identical potency (Fig 9).

The assay therefore provides an alternative and totally specific method to the autoanalyser technique for quantitating anti-D in prophylactic preparations. It also provides a means for comparing different batches of monoclonal preparations for red cell binding activity.

4.1.3 Characterisation of Human Monoclonal Antibodies against Blood Group Antigens

In recent years many stable cell lines secreting antibodies with blood group specificity have been established from EBV-transformed lymphocytes from allo-immunised human donors; in some cases fusion with mouse myeloma cells has been carried out to form heterohybridomas. Such human monoclonal antibodies have become important grouping reagents, particularly for Rh antigens which are non-immunogenic in mice; those of IgG class against the Rh D antigen also have considerable potential for replacing polyclonal anti-D for the immune prophylaxis of haemolytic disease of the newborn since the success of anti-D prophylaxis and the hazards associated with deliberate immunisation of volunteers has resulted in a shortage of polyclonal anti-D in the UK. In selecting such antibodies, it is important to have a full picture of their immunochemical characteristics. Over the last few years it has become apparent that a particular feature of monoclonal antibodies is their ability to participate in unexpected cross-reactions not shown by polyclonal antibodies ie they demonstrate multispecific properties.

4.1.3 (a) **Multispecificity and Comparison with Natural (Auto)Antibodies**

Although the D antigen is believed to be expressed only on human erythrocytes, previous immunochemical studies within the Division of 6 human monoclonal anti-D antibodies showed that 3 cross-react with human and animal tissues. The IgG mab Fog-1 and UCHD4 cross-reacted with smooth muscle, whereas the IgM mab Mad-2 was found to recognise the intermediate filament protein vimentin which is structurally dissimilar to the Rh D polypeptide. The possibility that Mad-2 and other human mab against blood group antigens could be multispecific was further investigated in collaboration with Dr K Thompson and others at the University of East London using a much larger panel of 44 IgM and 28 IgG antibodies with specificities for A, Rh D, Rh C, Rh c, Rh E, Rh e, Jk^a and Jk^b. Culture supernatants containing the antibodies were screened by indirect immunofluorescence of tissue sections (rabbit stomach, kidney and

heart, and rat brain) and a rat glioma cell line C6, ELISA using a panel of foreign and autoantigens, and for rheumatoid factor-like activity using rabbit IgG-coated sheep erythrocytes, human IgG-coated latex beads and by ELISA.

Twenty-six (59%) of the IgM mab reacted with tissue components. The components most commonly recognised were smooth muscle, the ependymal cells lining the ventricles of brain, and astroglia. Others included fibroblasts, endothelial cells, cardiac muscle, kidney tubules and collecting ducts and cell nuclei. With one exception, the tissue-reactive antibodies bound to more than one cell type. With the exception of the 2 anti-A antibodies, tissue reactivity was associated with all the blood group specificities. However, none of the mab shared identical immunofluorescence profiles and there did not appear to be any correlation between blood group specificity and distribution of tissue staining. Absorption experiments confirmed that the tissue reactivities were associated with the red cell binding fraction of the culture supernatants. Many of the mab were subsequently shown to bind to the intermediate filament proteins vimentin and glial fibrillary acidic protein.

Twenty-two (50%) of the 44 IgM mab reacted with fixed monolayers of the rat glioma cell line C6. Seventeen of these antibodies were tissue-reactive. The structures appeared to be intracellular and included intermediate (vimentin) filaments, stress fibres, microspikes, mitochondria and secretory vescicles (Fig 10). At least 8 mab bound to more than one of these structures.

Using ELISA, 25 IgM mab (57%) reacted with one or more of the antigens tested (DNA, thyroglobulin, tetanus toxoid, haemoglobin, DNP and hen egg lysozyme), and 11 showed rheumatoid factor-like activity in at least one of the 3 assays used.

Of the 28 IgG mab, all with Rh specificities, only 6 (21%) reacted with tissue components and in every case the components recognised were smooth muscle, ependymal cells and/or astroglia. Only one of the mab reacted with C6 cells, and only 6 reacted with one or more antigens in the ELISA. Eight IgG mab (29%) demonstrated RF-like activity.

In summary, 84% of the IgM mab and 46% of the IgG mab showed striking cross-reactions with one or more unrelated antigens in different assays. In view of the diverse nature of the antigens recognised, and the lack of correlation between blood group specificity and distribution of tissue staining, it was concluded that IgM mab against blood group antigens are commonly multispecific whereas IgG mab with the same blood group specificities show less frequent and more restricted cross-reactions. Thus antibodies produced in response to exogenous antigens can display multispecific properties in common with natural (auto)antibodies. However, demonstration of multispecificity clearly depends on the number of antigens used to screen against.

While the above study was in progress, the D-binding fraction of human antisera and immunoglobulin preparations was re-examined for similar tissue cross-reactions, but none was found, providing further evidence that the tissue

cross-reactions shown by many anti-D mab occur through additional binding abilities of the mab rather than through structural homology between D and tissue components. During the course of these studies, however, it became apparent that the cell types commonly recognised by IgM anti-blood group mab were those commonly recognised by IgM antibodies in normal human sera: smooth muscle, ependymal cells, astroglia, cardiac muscle, collecting ducts in kidney and cell nuclei. Similarly, the cell types recognised by IgG anti-D mab were also recognised by IgG antibodies in normal sera (smooth muscle and astroglia). These results, together with those above, show that some IgM and IgG anti-blood group mab share specificities with natural (auto)antibodies.

4.1.3 (b) Demonstration of Autoreactivity by a Human Monoclonal IgG Anti-D with Clinical Potential

As already mentioned, human monoclonal anti-Rh D antibodies of IgG class have considerable potential for *in vivo* clinical use and 2 such mab, Fog-1 and Brad-3, have recently been tested for their ability to clear erythrocytes *in vivo*. Previous immunohistochemical studies have shown that Fog-1 cross-reacts with human and animal smooth muscle. Since this initial study was restricted to a small number of human tissues, further immunochemical studies of the specificities of purified Fog-1 and Brad-3 immunoglobulins (provided by BPL) were undertaken using animal tissues and a wide panel of human tissues from nearly 50 unrelated donors, most of whom were of known Rh D phenotype.

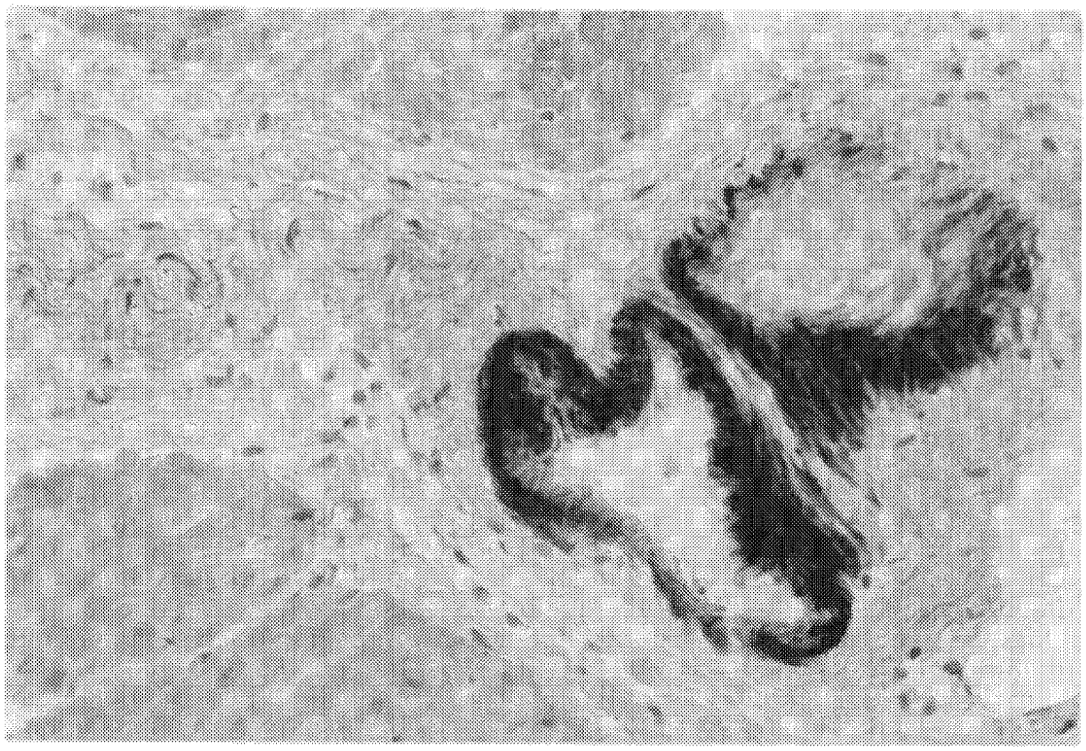
One of the mab, Brad-3, an IgG3, showed dose-dependent binding to multiple tissue components, whereas the other mab, Fog-1, an IgG1, consistently bound to animal and human smooth muscle (vascular, in the walls of hollow organs, in the respiratory tract) from both D-positive and D-negative donors (Fig 10). Fog-1 also reacted with stratified squamous epithelia, Hassall's corpuscles in thymus, and some glial processes. Immunoprecipitation experiments indicated that the smooth muscle epitope was actin or actin-associated.

Fab fragments were prepared from Fog-1 and Brad-3 by papain digestion. The ability to bind to smooth muscle was retained by the Fog-1 Fab fragments, but no significant tissue-reactivity was shown by the Brad-3 Fab fragments, indicating that the dose-dependent tissue-binding shown by the intact antibody was mediated by regions of the molecule well outside the Rh D antigen-binding site. The affinity constant for Fog-1 binding to smooth muscle sections was estimated to be about $1.2 \times 10^7\ M^{-1}$, which is over 2 orders of magnitude lower than that reported for Fog-1 binding to Rh D ($2.2 \times 10^9\ M^{-1}$). In a direct comparison of the affinity of Fog-1 for Rh D and smooth muscle, the latter was unable to compete with D-positive erythrocytes for Fog-1. The finding that smooth muscle homogenate did not inhibit binding of Fog-1 to erythrocytes suggested that the smooth muscle cross-reaction may not occur at the Rh D antigen-binding site, but through another part of the Fab portion.

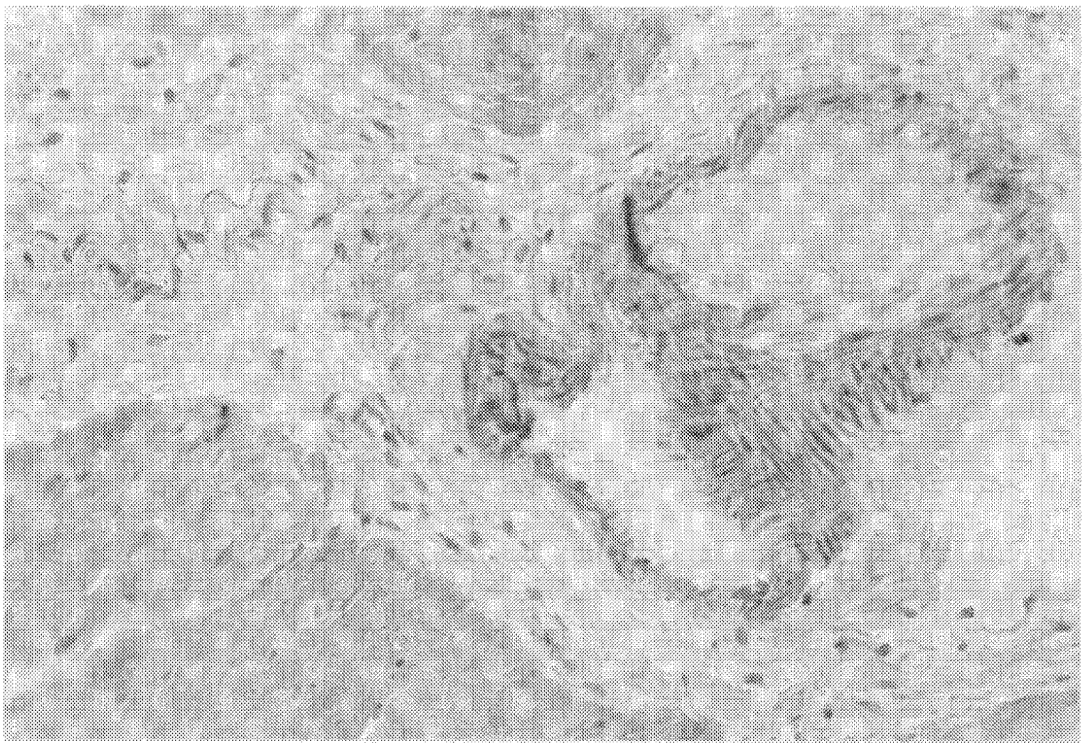
Although it could be argued that relatively low affinity cross-reactions with intracellular antigens are unlikely to cause clinical problems, the consequences can only be determined with certainty following *in vivo* use.

Figure 10 Alkaline phosphatase immunostaining of human heart tissue using the IgG anti-D monoclonal antibody FOG-1 (A). The red immunostaining is localised to vascular smooth muscle. The negative control is also shown for comparison (B).

A



B



The conclusion from this study was that cross-reactions revealed by *in vitro* testing can indicate where potential problems may occur, which can then be investigated for, and can provide possible explanations if *in vivo* use is unsuccessful, but will not necessarily predict *in vivo* consequences with certainty. In addition, the relative ease and inexpense of *in vitro* testing for undesirable cross-reactions can also facilitate selection of suitable mab for clinical trials.

4.1.3 (c) Characterisation of further Human Monoclonal Anti-D Antibodies with Clinical Potential

A number of laboratories around the world have produced human monoclonal IgG anti-D which have been offered to the Division for assessment of tissue-reactivity.

1. Stable cell lines secreting human anti-D mab have been established by EBV-transformation of peripheral blood lymphocytes from immunised donors, in some cases followed by fusion with a mouse myeloma line to form heterohybridomas. Multireactive IgG anti-D mab have been produced by both methods. The tissue-reactivity of an IgG monoclonal anti-D, ESD4, provided by Dr Moira McCann-Carter of the SNBTS, was assessed using culture supernatants from the EBV-transformant and from the same clone after fusion to form a heterohybridoma. Interestingly, tissue-reactivity (binding to smooth muscle) was found in the latter but not the former. Some changes in reactivity with certain D categories (tests performed by the SNBTS) were also found. The possibility that the observed change in properties was due to altered glycosylation was examined by screening culture supernatants from the heterohybridoma grown in tunicamycin. However, this procedure did not alter or abolish the tissue-reactivity of the heterohybridoma antibody indicating that this property is probably not mediated by carbohydrate moieties on the antibody.

2. The SNBTS and CRTS in France are currently evaluating a large number of human monoclonal anti-Ds for clinical use.

Again in collaboration with Dr Moira McCann-Carter of the SNBTS, 21 anti-Ds, including 2 from Lille which are currently undergoing clinical trials, have been screened for undesirable cross-reactions with human tissues.

3. A third BPL anti-D, Brad-5, has also been tested for undesirable cross-reactions with human tissue antigens. This anti-D is an IgG1 and is currently undergoing clinical trials. Like Fog-1, Brad-5 also cross-reacted with smooth muscle, stratified squamous epithelia and Hassall's corpuscles.

4. About 30 culture supernatants containing monoclonal IgG anti-D from Dr Belinda Kumpel in Bristol and Dr Anne Fletcher in Australia have been tested for cross-reactions with animal tissues.

4.1.3 (d) **Isoelectric Focusing of Monoclonal Antibodies**

It has been suggested that multireactivity is mediated by charge interactions. In view of a report that polyclonal anti-D has a relatively high pI and our finding that many of the multireactive anti-blood group mab were found to bind to the acidic proteins vimentin and glial fibrillary acidic protein, the pIs of a number of human monoclonal antibodies are currently being measured to determine whether there is a correlation between antibody pI and specificity or multireactivity. To date, the pIs of 14 IgG mab with specificities for Rh antigens, tetanus toxoid and IgG (the latter are RF produced from patients with rheumatoid arthritis) have been determined using agarose gels. The antibodies displayed varying degrees of microheterogeneity, and had pIs in the range 8.5 to 9.7 (slightly more basic than polyclonal immunoglobulin), but with no clear correlation between pI, multireactivity, subclass or specificity.

Microheterogeneity is caused, in part, by glycosylation differences. Glycosylation of antibody is reported to be necessary for effector function, and it is known that differences in cell culture methodology can result in differences in glycosylation. Since it is thought that anti-D immunoglobulin prevents maternal sensitisation to D-positive red cells by mediating their rapid clearance from the mother's circulation and destruction, glycosylation of mab produced *in vitro* that are intended for *in vivo* use is an important consideration. It is therefore proposed to use IEF in conjunction with labelled lectins to study glycosylation of mab.

4.1.3 (e) **Comparison of Properties of Anti-blood Group mab with Human Monoclonal RF and Correlation with V Gene Usage**

There is currently much interest in the use of human monoclonal antibodies for studying the autoantibody response in autoimmune and other diseases. However, our recent study of the specificities of human mab against blood group alloantigens, derived from healthy immunised donors, showed that many of the mab were able to cross-react with one or more normal tissue components, some of which are target antigens recognised by antibodies present in autoimmune syndromes. To gain insight into the significance of tissue reactivities and to determine whether mab against auto- and allo-antigens have distinct or overlapping tissue reactivities, those of a panel of monoclonal RF, established from patients with rheumatoid arthritis are currently being investigated in collaboration with Dr K Thompson. The findings from this study and the anti-blood group antibody study will also be correlated with VH gene usage by the mab in order to determine whether multispecificity is associated with the use of particular V genes. These results may be relevant to the use of CDR grafting to 'humanise' mouse monoclonal antibodies as well as to the study of autoimmune syndromes.

4.1.3 (f) **Characterisation of Erythrocyte-reactive Fv Fragments**

The technology now exists to produce human antibody fragments from heavy and light chain variable gene repertoires. Immunohistochemical studies of one

such erythrocyte-reactive Fv fragment produced by Dr J Marks and others at Cambridge and Dr W Ouwehand have shown that the fragment has blood group B specificity. It bound to the endothelia and epithelia of tissues from blood group B donors, but not to those of blood group A and O donors.

It is hoped to expand work in this area by using similar technology to swap heavy and light chains of anti-blood group antibodies to assess their relative contributions to the properties eg specificity, affinity, of antibodies.

4.2 PLATELETS

Following the appointment of Dr W Ouwehand on a part-time basis the intention was to initiate work in 2 areas:

i) platelet immunology, with particular attention to detection of clinically important antibodies; ii) quality of platelet concentrates. Although a scientific officer was transferred to work in this area, difficulty was experienced in recruitment of a post-doctoral scientist, and the work on platelet concentrates was not started. The current situation is that the scientific officer left 6 months ago and was not replaced; a tenure-track scientist, Dr Paul Metcalfe, has now been appointed and joined the Division on 1 November 1993. The initial development phase of the platelet immunology project has now been completed and work will now commence on investigation of markers of platelet activation which could be applied to monitor the quality of platelet concentrates.

4.2.1 Platelet Immunology

Background

Allelic variation of genes encoding several platelet glycoproteins has been described and is at the basis of platelet specific blood group systems. The allelic variation of platelet glycoprotein IIIa, Ibd, IIb and Ia can lead to the formation of harmful alloantibodies after a transfusion or in pregnancy. In the former the alloantibodies against platelet glycoproteins can reduce the survival of donor platelets which are positive for the relevant alloantigen (platelet refractoriness). On rare occasions the boosting of alloantibody formation might remove the immune response restriction for self and lead to the destruction of the patient's own platelets as well as the donor platelets resulting in the clinical syndrome of post transfusion purpura (PTP). In pregnancy maternal IgG alloantibodies against platelet glycoproteins can cause severe thrombocytopenia in the foetus with a risk of cerebral haemorrhage in the perinatal period. Thus the detection and identification of platelet specific alloantibodies is of clinical relevance.

Two projects are related to the above:

4.2.1 (a) Restriction Fragment Length Polymorphism Analysis (RFLP)

RFLP analysis has been used to genotype donors for the clinically relevant

platelet specific blood groups (the alloantigens HPA1, 2 and 3 systems). In collaboration with the North London Blood Transfusion Centre and the Division of Transfusion Medicine (DTM) at the University of Cambridge around 500 donors have been genotyped for HPA1, 2 and 3 using RFLP analysis. Genomic DNA was amplified with the appropriate sets of primers flanking the relevant point mutation in the genes of glycoprotein IIIa, Ibα and IIb, respectively.

Selected donors were phenotyped for HPA 5 using the monoclonal antibody immobilisation of platelet glycoprotein assay (MAIPA) and reference sera were obtained from Professor C Müller Eckhardt in Giessen, Germany.

Based on the above results 6 donors have been selected and enrolled on a monthly platelet cytapheresis programme. The donor platelets have been cryo-preserved and are made available as a 3 cell panel for the detection of platelet specific alloantibodies. So far 8 centres in the UK are using this reference panel. The quality of the panel has been assessed in a National Workshop with 30 participating laboratories and the platelets of 6 donors have been retyped by an external reference laboratory (CLB of the Dutch Red Cross BTS, Department of Immunohaematology, Amsterdam, The Netherlands).

4.2.1 (b) **Provisional NEQAS**

Four workshop-type surveys have been organised from NIBSC to analyse interlaboratory variation in the detection and identification of platelet specific alloantibodies. The surveys highlighted several problems:

i) enormous variations were observed between laboratories in the sensitivity of the assays used for the detection of platelet specific alloantibodies

ii) more than half of the laboratories are using platelet cell panels which are inappropriate for the detection of the clinically relevant platelet specific antibodies, and

iii) several participants frequently report false positive results.

A proposal to formalise these surveys into an official NEQAS for the detection of platelet reactive alloantibodies, which in its outline would be very similar to but less frequent than the red cell serology scheme, has been approved by the Department of Health. The platelet NEQAS would be supervised by the Steering Committee with Mrs Judith Chapman (Department of Haematology, St Bartholomew's Hospital, London), Mr Dave Allen (RTC, Oxford) and Dr Peter Phillips (NIBSC) with Dr W Ouwehand as Chairman.

4.3 BLOOD GROUP SEROLOGY

4.3.1 UK National External Quality Assessment Scheme for Blood Group Serology (UK NEQAS (bgs))

4.3.1.1 Introduction

A UK NEQAS exists for each of the principal areas of clinical pathology. Their objective is to assure the adequate performance of clinical laboratories by interlaboratory comparison of the results on coded exercise material. UK NEQAS is an adjunct to a participant's own internal quality management systems. The objective is to improve the performance of underachieving participants, primarily through education not punition.

Participants who incur critical errors, the definitions of which are approved by a committee (Joint Working Group) drawn from the relevant professions, are 'unsatisfactory performers'. Participants with 2 or more unsatisfactory performances in 4 consecutive exercises are 'persistent unsatisfactory performers'. Mechanisms exist for the Scheme Organiser to contact unsatisfactory performers to offer appropriate assistance. Continuing unsatisfactory performance is addressed by the Joint Working Group, who report to the Secretary of State for Health.

Prior to 1991/92, UK NEQAS were funded by grant from the UK Department of Health. With the exception of the histopathology and certain pilot schemes that continue to be funded by the Department, the UK NEQAS are now funded totally by the participants' subscriptions. With the exception of the administrative fee charged by a host authority for accommodating the Scheme's organising laboratory, the monies received by the Schemes are for the benefit of that Scheme; any excess is returned to the participants via future subscription fees. The finances are audited annually by the UK NEQAS Executive.

Pending the development of a coherent strategy for external quality assessment, which will include the introduction of competing schemes within the larger specialities, the Department has devolved the oversight of UK NEQAS to a UK NEQAS Executive, the members of which are elected by the individual Scheme Organisers. Although participation in UK NEQAS is voluntary, UK accreditation of clinical laboratories by Clinical Pathology Accreditation Ltd (CPA) requires participation in relevant approved external quality assessment schemes. In addition, purchasers of clinical laboratory services often specify adequate performance in approved external quality assessment schemes. UK NEQAS (bgs) has applied for recognition as an approved external assessment scheme for CPA accreditation purposes.

Each Scheme has a Steering Committee comprising established members of the relevant professions, chaired by an individual of national status. The objectives of the Committees are:

- to provide support to the Scheme Organisers;
- to ensure that exercises address the important issues; and
- to ensure that exercises are fair and are issued at appropriate intervals.

The Committees meet regularly and review:

- the general performance of participants;
- the ability of exercises to discriminate between adequate and unsatisfactory performers; and
- the operation of the Scheme.

The Scheme Organiser for UK NEQAS (bgs) is Dr Susan Knowles, Medical Director of the South Thames Regional Blood Transfusion Centre.

NIBSC was invited to host the organising laboratory and manage the Scheme because of its central role within the UK together with the long-established expertise of the Head of Standards Division who is the current Scheme Manager for UK NEQAS (bgs) in the field of blood group serology.

4.3.1.2 Principle of Operation

Participants register to participate in various sections, according to the work routinely undertaken in their laboratory:

- ABO and RhD grouping;
- antibody screening;
- antibody identification; and
- compatibility testing.

There are 10 exercises distributed annually. Four of these exercises comprise:

- 3 'patient' paired samples of red cells and serum; and
- 3 'donor' red cell samples.

Participants are asked, according to their registration, to ABO and RhD group, determine and identify the presence of alloantibody and determine the compatibility of the 'donor' red cells samples with the 'patients' samples, using their routine procedures.

The other 6 exercises comprise up to 6 'patient' serum samples. Participants are asked, according to their registration, to determine and identify the presence of alloantibody.

The results and their interpretation are returned to the organising laboratory where they are analysed against the 'correct' expected result. A report is returned to the participant usually within 5 days of the closing date for the exercise. This report details an individual participant's results against that of the 'correct' expected result and summarises the performance of the UK as a whole in the various sections of the exercise. Some exercises are accompanied

by a questionnaire, the returns from which enable the organising laboratory to analyse any link between performance and the techniques undertaken. These detailed reports are returned to participants with the next distributed exercise.

4.3.1.3 The 'Correct', Expected Result

The 'correct', expected result against which a participant's results are assessed is a key determinant in the exercise. Currently, the 'correct', expected result is defined as that obtained by 80% or more of UK participants. However, with some samples consensus cannot always be achieved, and the Steering Committee is considering redefining the correct, expected result as that from 3 stated laboratories; the organising laboratory, a transfusion centre and a major hospital.

4.3.1.4 Participants

Participants are drawn from UK clinical laboratories -private and public sector, including trust status; manufacturers of equipment and reagents; and laboratories of the UK armed forces, at home or oversees. See Table 1.

It is seen from the table that there are 37 WHO sponsored participants. These are shown in Table 2.

These WHO sponsored participants are nominated by WHO to receive exercise material in order to stimulate the development of a central expert facility within that country. The organising laboratory within NIBSC provides various information to these WHO sponsored participants, for example, on reagent specification, suppliers, training guides and professional guidelines. The Institute has been nominated as a WHO Collaborating Centre for External Quality Assessment in blood group serology, for its work in this arena.

4.3.1.5 Staffing

The staff of the Organising Laboratory comprises 2 Scientific Officers, with a vacant post, totally funded by the Scheme. The Head of Standards Division devotes some 8% of his time to UK NEQAS, primarily on devising exercises in conjunction with the Steering Committee, reviewing preliminary data analysis and writing the exercise reports.

4.3.1.6 Reduction in Participants' Unsound Working Practices

Antibody Detection. Enzyme Tests

The Scheme has been successful in reducing the use of one-stage mix enzyme tests. Several exercises have clearly demonstrated the poor performance of these tests, in comparison with two-stage tests. Over the period from 1988 there has been an improvement in the detection of a reference anti-D preparation associated with the reduction in the use of one-stage mix tests, to 10% of enzyme test users.

Table 1 Participants in UK NEQAS (bgs)

Area of work	Number of participants
UK public sector, including trust status	360
UK private sector	85
UK Blood Transfusion Centres	34
UK Ministry of Defence, within the UK	5
UK Ministry of Defence, overseas	7
Manufacturers	2
UK non-clinical (reference)	4
Non-UK non-clinical (reference)	6
Non-UK hospital	25
WHO sponsored participants	37
TOTAL	565

Table 2 WHO Sponsored Participants in UK NEQAS (bgs)

South and Central America	
Jamaica	Trinidad and Tobago
Dominica	Monserrat
St Vincent	Bogota
Antigua	St Lucia
Brazil	Guatemala
Mexico (3)	Cuba
Venezuela (2)	Anguilla
Costa Rica (2)	Turks and Caicos Islands
Bahamas	St Kitts
Barbados	
Cayman Islands	
Africa	
Rwanda	Madagascar
Bangui	
Western Pacific	
Malaysia	China (3)
South East Asia	
Indonesia	Sri Lanka
Korea	Thailand
Middle East	
Israel (2)	

The Manufacturer's Recommended Method

Surveys have highlighted a number of deviations from manufacturers' recommended methods, eg in the use of antiglobulin reagents; such deviations may invalidate the manufacturers' liability should an incorrect result be obtained. The educational elements of the Scheme have emphasised the importance of following the manufacturer's recommended techniques. Instances have been encountered where the manufacturer's recommended technique has been unsatisfactory. Discussions with the manufacturers have resulted in the recommendation by the manufacturer of a more appropriate technique.

4.3.1.7 **Educational Aspects of the Scheme**

A series of one-day meetings is held; usually on a Saturday when over 200 participants attend. Members of the Organising Laboratory, together with other leading figures in the field, present lectures to stimulate discussion on such issues as newly introduced technologies, reagent specifications and on the continuing use of clearly unacceptable methodologies. The British Blood Transfusion Society has agreed to publish the proceedings of these meetings.

Participants who are experiencing difficulties are encouraged to refer to their Transfusion Centre. Where, for some reason, this is not acceptable or fruitful, participants are invited, on a confidential basis, to the organising laboratory and to bring the reagents and consumables used in their laboratory. They undertake serological examinations in parallel with staff from the organising laboratory. In this way, the reason for poor performance often can be identified.

4.3.1.8 **Data Handling**

The Organising laboratory has invested in Optical Mark Readable (OMR) technology. This has enabled data previously requiring some 2 weeks to be entered within 2 hours. Whereas this OMR technology has enabled the staff of the Laboratory to increase its efficiency, the forms are noticeably different from those used by the participants on a day-to-day basis. This may be the contributory factor to some transcription errors.

Constructive discussions have been held with the Head of Informatics at the Institute on alternative means of obtaining the participants' data, and various proposals are still under consideration.

4.3.2 Reference Preparations for Blood Grouping Reagents

Where defined, the US FDA minimum potency preparations have been the *de facto* standards for blood grouping reagents. Since these preparations are available in general only to manufacturers with a clear intention to supply into the US market, it is difficult for many EC manufacturers to compete with US manufacturers. Furthermore, there is no requirement for US-based companies to comply with US requirements for batches exclusively supplied to the non-US

market and there is some data to suggest that sub-US standard reagents have been 'dumped' within the EC.

The Guidelines for the Blood Transfusion Service refer to preparations of agreed minimum acceptable potency and these are now being produced. Reference preparations of agreed minimum potency have been prepared for anti-A and for anti-B blood grouping reagents. Comments are being sought from interested parties on the proposed anti-D reference preparation following completion of the stability testing. Also available is a minimum potency preparation for rabbit complement used in HLA class I serology which has significantly improved the quality of results in this area.

Where relevant, these British reference preparations have been made to an equivalent potency to the US minimum potency preparations, since reagents' manufacturers supply a world-wide market and therefore a single potency standard is attractive to them. The US FDA is aware of this programme and has contributed the US minimum potency preparations, where relevant. The proposed anti-D reference preparation was assessed at an international meeting, held under the auspices of the International Committee for Standardisation in Haematology (ICSH) and International Society for Blood Transfusion (ISBT) at which delegates from EC and other European countries, Japan, Australia and the US attended. Following an initial evaluation by EC observers at the international meeting referred to above, funding may become available from the EC BCR programme to produce equivalent reference preparations for use within the EC.

Under the auspices of the ISCH/ISBT, a defined protocol for the assay of proteolytic enzymes used in blood group serology has been developed and a reference preparation of papain produced at a potency which balances activity against freedom from false-positive reactions. This will soon be available for distribution.

4.3.3 Other Reference Preparations

Studies by the UK NEQAS organising laboratory and others have shown that, in general, the major factor in the performance of a critical test such as the antiglobulin test is the performance of the operator in detecting weak but definite agglutination, and the efficacy of the red cell washer in removing protein prior to the addition of the antiglobulin reagent. In conjunction with Dr Voak (East Anglia Blood Transfusion Service) and others, an anti-D preparation has been developed to address these issues. The antibody is naturally weak and effects agglutination that readily disintegrates with excessive mechanical trauma. Its dilution for use was determined after extensive multicentre trials. It is distributed with protocols to enable laboratory staff to assess the efficacy of red cell washers in removing protein and of operators in detecting weak agglutination. One recipient volunteered that the material demonstrated a false-negative rate in the antiglobulin test of 30%, which was reduced to 2% with staff training over a 2-week period.

4.3.4 Assessment of New Technologies

A significant change in serological testing has been the introduction of micro-column methods, where agglutinates are separated from unagglutinated red cells on centrifugation by a dextran gel or glass beads. Early papers showed that the micro-column tests were more sensitive than conventional antiglobulin test. However, careful studies in the UK NEQAS Organising Laboratory and elsewhere showed that the initial technology had a sensitivity, in general, comparable to spin-tube methods undertaken with a competent, assured operator. In addition, the micro-column method was less sensitive with certain antibody specificities, particularly with heterozygous test red cells, as may be encountered in the crossmatch. Discussions with the manufacturers have led to improvements in the antiglobulin reagent and a more realistic claim of performance. The enzyme method is reported to detect antibodies that are of no clinical significance and this is being investigated.

A grant has been awarded to the Blood Transfusion Task Force for Dr D Voak in conjunction with Dr Marion Scott at the International Blood Group Reference Laboratory (Bristol) and the Organising Laboratory, to investigate 3 new serological technologies on behalf of the Procurement Division of the Department of Health.

4.4. GENERAL HAEMATOLOGY

4.4.1 Ferritin

Standardisation

The concentration of serum ferritin reflects the level of iron stores and the assay of serum ferritin has therefore become widely used to diagnose iron-related disorders and in population surveys of iron status. A number of ferritin preparations have previously been subjected, by the ICSH, to a collaborative study in which 2 preparations, 80/602 and 80/578, were shown to be suitable as reference materials for the serum ferritin assay. Both preparations were assigned a ferritin content of 9.7 ug/ampoule, and appeared similar to each other and to local standards. Both showed satisfactory stability. 80/602 was subsequently established as the IS, but stocks are nearly exhausted. In collaboration with Dr M Worwood of the University of Wales College of Medicine and Mr A Heath of Informatics, NIBSC, a limited, comparative study of 80/602 against 80/578 was carried out to provide validation of the results of the original study. Six international laboratories took part. The results validated the value of 9.7 ug/ampoule assigned in the original study, and WHO established 80/578 as the 2nd IS in 1992.

In collaboration with Dr Worwood, a recombinant ferritin preparation is now being assessed as a potential replacement of 80/578.

4.4.2 Haemoglobin

The diagnosis of ß-thalassaemia trait (heterozygous ß-thalassaemia) relies largely on raised levels of the minor adult haemoglobin Hb-A_2 and, in about half of affected individuals, fetal haemoglobin, Hb-F, which normally comprise about 2.2%-3.2% and 0.5% of the total haemoglobin in adults respectively. Measurement of the former follows its electrophoretic separation from the major adult haemoglobin Hb-A on cellulose acetate strips and subsequent elution; the latter is measured following alkali denaturation of the adult haemoglobins.

Standardisation

Levels of Hb-A_2 above 3.8% are indicative of ß-thalassaemia trait but there is a degree of diagnostic uncertainty if the level falls between 3.2-3.7%. The alkali denaturation test can give inconsistent results particularly in the low range. It was therefore felt that international reference preparations of haemolysate containing normal and raised levels of Hb-A_2 and Hb-F would be useful for laboratories carrying out these measurements. Materials were ampouled at NIBSC, and Drs A Stephens and B Wild of St Bartholomew's Hospital organised an international collaborative study. Following assistance and advice from the Division (and from Mr A Heath of Informatics), 2 materials have now been offered as standards to the WHO.

Associated research

The distribution of the haemoglobin content of erythrocytes is also important in the study and diagnosis of haemoglobinopathies. In adults, Hb-F is restricted to a subpopulation of erythrocytes termed F cells. Generally there is good correlation between the amount of Hb-F and the proportion of F-cells, but due to the inconsistency of the alkali denaturation test at low Hb-F levels, F-cell number is usually the parameter of choice in the diagnosis of some haemoglobinopathies such as heterocellular HPFH which are characterised by small increases in levels of Hb-F. A simple fixation and permeabilisation procedure was previously developed which allows erythrocytes to be labelled in suspension with antibodies against haemoglobin for FACS analysis. In a collaborative project with Dr S L Thein of the MRC Molecular Haematology Unit, the method has been used for immunochemical estimation of Hb-A and Hb-F and their distribution in red cells from normal individuals and individuals affected with haemoglobinopathies.

4.4.3 B12 and Folate

B12 and folate deficiencies are a common cause of disease such as pernicious and megaloblastic anaemia respectively.

The 1st British Standand for human serum vitamin B12 was upgraded to an IRR by WHO in 1992.

The Division has been collaborating first with Dr D W Dawson and more

recently with Dr Sourial of St Bartholomew's Hospital over the preparation of a red cell folate standard. Two methods are used for folate measurement: a microbiological assay and a radioisotopic method for which commercial kits are available. Although material ampouled in 1990 was subjected to a collaborative study, it did not show acceptable long-term stability using the microbiological method. Trial fills in which a stabiliser will be included are now planned for the near future.

4.5 FUTURE DEVELOPMENTS

4.5.1 Immunohaematology

Standardisation

i) Recombinant ferritin will be evaluated as a possible replacement of ferritin isolated from human tissues.

ii) Work will continue on the production of a stable folate preparation.

iii) Potencies of batches of anti-D immunoglobulin submitted to the Institute for batch release will be determined.

iv) Techniques to evaluate intact effector function of monoclonal anti-D antibodies should be established. This aspect is equally important to evaluating the red cell-binding activity if the antibodies are to be used successfully in the prevention of HDN.

Associated Research and Development

Characterisation of monoclonal antibodies

i) Since screening mabs against human tissues for undesirable cross-reactions is amongst the 'points to consider' in both the EC and FDA guidelines relating to the *in vivo* use of mabs in humans, and because of the Division's expertise in immunohistochemistry, the Division has been asked by Quality Biotech to carry out immunohistochemical surveys of human tissues using mabs undergoing clinical evaluation on a commercial basis. It is hoped that this will eventually lead to the funding of a new post. This work will also provide the Division with the opportunity to gain experience with chimeric and humanised mabs as well as with rodent and human mabs.

ii) It is hoped to use molecular biology techniques to study the effect of light chain swapping on the properties of anti-blood group mabs. Gaining expertise in such methodology would seem desirable in view of the use of molecular biology to construct antibodies for clinical use.

Haemoglobin

Accurate measurement of Hb-A_2 is important for diagnosis of haemoglobinopathies. It is proposed to attempt to produce a monoclonal antibody specific for the δ-chains of Hb-A_2 for development of new methods for Hb-A_2 detection which can also be applied to the study of haemoglobinopathies.

4.5.2 Platelets

i) The RFLP technology is based on the digestion of DNA with restriction enzymes. Besides the technical problem of incomplete digestion which could lead to mistyping also the costs of the assay are rather high and the testing is labour intensive. Allele specific primers are being designed and the PCR conditions are altered in such a manner that the allele specific primer will only bind to the template of one allele but not the one of the antithetical allele. For the point mutation in the GpIIIa gene this approach has already proven to be successful and now efforts are made to establish also this for HPA2, 3 and 5 (mutations in the GPIbα, GPIIb and GPIa gene, respectively). As soon as the sequence of the intron at the 5' end of the point mutation in GPIa gene has been determined a similar approach will be followed to design an allele specific PCR for the HPA5 system.

ii) Freeze-dried Weak Anti-HPA1a

Anti-HPA1a is the most frequently occurring platelet-specific alloantibody causing clinical disease. Therefore, a freeze-dried preparation has been prepared from human plasma containing a low level of anti-HPA1a antibody. This preparation will be evaluated in the forthcoming platelet QAS as a weak positive standard. This standard can then be used to monitor laboratory performance.

iii) Monoclonal Antibodies (mabs) against Platelet Glycoproteins

Several platelet specific alloantibodies are only detectable in the MAIPA assay and not in the immunofluorescence test. For the MAIPA test murine monoclonal antibodies are needed to purify specifically the different alloantigen carrying platelet glycoproteins from a platelet lysate.

To improve and standardise the detection of platelet specific alloantibodies freeze-dried monoclonal antibodies against GpIIb/IIIa (CD61), GPIa/IIa (CD49) and GPIb/IX (CD42) will be made available during 1994.

iv) Through its collaboration with the Department of Transfusion Medicine at the University of Cambridge the Division will be involved in 3 related projects.

a) Cloning of anti-HPA 1a and 1b antibodies by phage display

technology, structure/function and possibly crystallisation studies of GPIIIa

b) Prospective clinical study in 30 000 pregnancies to determine the morbidity and mortality caused by anti-HPA1a in foetuses/neonates.

c) Development of assays for the detection of anti-HPA1a and 1b alloantibodies using recombinant GPIIIa leucine 33 and GPIIIa proline 33.

v) Platelet Concentrates

The general approach will be to study platelet activation and the platelet storage lesion in concentrates, with the aim of developing a test which can be used to relate to their clinical performance. Initial studies will use monoclonal antibodies to platelet glycoproteins and the thrombin receptor, as well as the phospholipid binding protein Annexin V.

FUTURE NEEDS OF THE DIVISION

1. OVERALL BALANCE OF WORK

It is envisaged that the balance of work among the 4 programme areas will remain broadly the same as at present, although as the development of an increasing variety of new antithrombotic drugs proceeds it is possible that greater emphasis will have to be placed in this area. The extent to which the Transfusion Medicine work continues and expands depends on the development of adequate funding agreements with the Transfusion Service, and the usefulness of the methodolgy and standardistion work currently in progress to the Transfusion Service. The Institute's work on Transfusion Medicine is somewhat fragmented, and although the blood virology work is probably best carried out in a virology context, a case could be made for integration of the blood group serology work carried out in the Standards Division with the more research and development oriented work on blood group antigens and platelets in the Division of Haematology, if the accommodation problems implicit in such an integration could be solved.

Two aspects of our control and standardisation work will be briefly considered since they may affect the future requirements for resources; European directives on batch control, and standardisation of diagnostic kits and tests.

Batch control

The implementation of European directive 75/319, which forbids batch release of biological products before marketing, with the exception of blood products and vaccines, has already had a considerable impact on the control activities of the Institute. In the Haematology Division, blood products account for the majority of the control work and will continue to be subject to batch release. However, the majority of antithrombotic drugs and thrombolytic agents do not fall into this category, and although batch release arrangements are continuing for the moment with currently licensed products, new products in these areas, as well as new recombinant clotting factors, will not be subject to batch control. The emphasis in future with these products will be on pre-licensing studies, followed by post-marketing surveillance. However, these altered arrangements are not yet in place, and in the opinion of the Head of Division, urgent consultation is required with the MCA to clarify and improve the present arrangements where we depend entirely on the manufacturers' goodwill for obtaining samples.

Diagnostics

Although historically most of the standards in the Institute have been established for control of therapeutic materials, the need for additional standards for diagnosis and control of treatment of patients was recognised very early in the Division of Haematology. The first British Standards for FVIII in plasma were established in the early 1970's and were followed by additional plasma standards, both national and international, for several other plasma clotting factors and inhibitors. These standards are used in clinical laboratories to calibrate assays in plasma for the diagnosis and

treament of haemorrhagic and thrombotic conditions, and because of the requirements of many laboratories for working standards to be used directly in assays, the British Standards are subject to very high demand, requiring frequent replacement (the current BS for FVIII in plasma is the 19th). Now that batch size has been increased so that replacement is required less frequently, the Head of Division considers that there are 2 additional areas where the expertise of the Division in this area could be helpful.

1) For the control of oral anticoagulants, there is a need for reference plasmas to supplement the reference thromboplastins already available. This is already being addressed at the European level through the EC funded project in the Division, and the possibility of establishing international reference plasmas will also be explored, through the ISTH/SSC; Dr Barrowcliffe has been invited to take over the Chairmanship of the SSC Sub-Committee on Anticoagulant Control in 1994. Similar reference plasmas for control of heparin therapy may also be useful and another area where NIBSC has been asked to help is in the provision of reference plasmas for lupus anticoagulant testing.

2) The evaluation of diagnostic kits, though not one of our statutory functions, has long been considered as a possible part of our remit, subject to available resources. In the area of haemostasis and thrombosis diagnostic tests and reagents are becoming increasingly complex, and NEQAS surveys indicate very high inter-laboratory variability, especially with some of the newer kits. Although this is potentially a very large area of work, useful contributions could be made with appropriate selections of topics, in consultation with professional bodies such as NEQAS and the British Committee for Standards on Haematology (BCSH), an example being the evaluation of FVIII deficient plasmas carried out by Dr Barrowcliffe and colleagues on behalf of the Haemostasis and Thrombosis Task Force of BCSH. Dr Barrowcliffe is a member of this Task Force and of the NEQAS Steering Committee, and is thus well placed to consider suitable evaluations which can be carried out in the future within the resources of the Division.

2. FUTURE SCIENTIFIC PRIORITIES

Future scientific work planned within each programme area has been described in detail in the report, and will be summarised only briefly here. In general, the emphasis will be on research and development relating to new products, particularly those of recombinant origin such as FVIII, tPA variants and new antithrombotic agents.

Blood Products

Work on the immunogenicity of FVIII will expand and the potential of human antibody production using the molecular genetic technique will be explored further. Control work on FVIII will probably diminish slightly as the number of batches continues to decrease, but work on the development and standardisation of the chromogenic assay will continue. The introduction of regular international collaborative studies on FVIII assays in concentrates for manufacturers and control authorities is currently being

considered. This could become a type of external quality assurance scheme, and apart from its scientific value could potentially bring in useful additional income. Fundamental studies on the biochemistry of FVIII and FIX will be extended, with the emphasis on an enzymology approach as developed by Dr Longstaff. Albumin control work has already been reduced and is proposed to continue at the present level.

Antithrombotic Drugs

Further *in vitro* studies on the action of LMW heparin are planned, with the emphasis on development of more physiological control techniques; studies of the effects of endothelial cells and platelets will be expanded. Research and standardisation work on protein C and TFPI will be extended. Consideration has also been given to the initiation of work on 2 other agents currently under development - recombinant thrombomodulin and monoclonal antibodies to platelet glycoproteins. These agents have different mechanisms of action from the drugs studied so far, operating via the endothelium and platelets, respectively, and new methodology will have to be developed for their control. This whole area is expected to be one of rapid growth in the near future, and if we are to carry out meaningful control, standardisation and research on all these agents, consideration will have to be given to increasing resources.

The project on cytokines, haemostasis and thrombosis is also potentially a large area of fundamental interest, and it is proposed to apply for external funding to continue this project. Discussions have been held with Dr Griffiths in the Division of Bacteriology about a possible collaborative project on the haemostatic complications of meningitis, but it is considered that resources are insufficient at the moment to pursue this area.

Thrombolytic Drugs

Standardisation and research work on tPA will be extended as new recombinant variants are developed, and similar studies will be carried out on the various types of SCuPA. The molecular biology approach to tPA, with the production of specific mutants for mechanistic studies, will be continued. Enzymology studies on various aspects of the fibrinolytic system will continue and be extended to other areas in the Division, notably heparin and FVIII. The emphasis in future studies will increasingly be on interaction at the cell surface, as well as in solution. Basic studies on the interaction of the fibrinolytic and coagulation systems in thrombolytic therapy are of considerable interest and external funding will be sought for this work.

Transfusion Medicine

Studies on anti-D will be extended to encompass other blood group antibodies, and the technology of antibody engineering will be explored to study antigen-antibody interactions in detail at the molecular level. Further work will be carried out to maintain the platelet reference panel and to develop the proposed platelet NEQAS, and the studies on markers of platelet activation in concentrates will be initiated. Another area of great potential importance in the future is the development of modified (cross-

linked) haemoglobin as red cell substitutes. Preliminary clinical trials have been carried out in the USA but have been suspended because of adverse reactions. Although no work is currently carried out on these products in the Division, discussions were held with Dr Griffiths of the Division of Bacteriology because of concerns about possible effects of haemoglobin on susceptibility to bacterial infection. With the help of a grant from the SNBTS, this was investigated and initial results with experimental material supplied by SNBTS indicated that modified haemoglobin was more effective than native haemoglobin in enhancing infection in experimental animals. Depending on the success or otherwise of these products, the Haematology Division may become involved in future in characterisation and control, eg monitoring the degree of cross-linking and polymerisation.

3. STAFF NEEDS

The existing number of 9 scientific staff is considered adequate to meet the current and short to medium term future needs. The balance of scientific interests and expertise among the scientists fits well with the needs of the Division, particularly since more flexibility has been introduced with scientists contributing to more than one programme area.

The number of support staff currently in post is 9, including one person on maternity leave, with 2 vacancies, excluding the EC funded scientific officer. Since earlier this year the Institute decided to apply for accreditation of its control testing on batch release of blood products and vaccines, Mr John Pring, our Laboratory Manager, was appointed Quality Co-ordinator in the Division. This has led to a considerable increase in his administrative duties and hence a decrease in the amount of his time spent on scientific work. At present therefore the number of support staff available for bench work is down to 7½.

The Head of Division considers that this is inadequate to meet our current demands, and requests that urgent consideration be given to replacement of the 2 vacant posts. The Division has a heavy load of control and standards work, and although the introduction of the ACL automated coagulometers has helped to streamline our control testing much of the work is still labour intensive. At times of staff shortage the more investigative aspects of the work inevitably suffer, since we must fulfil our control and standards commitments. Even when fully staffed there is little scope for expansion of our research and development programme. As already indicated, there is a growing need for additional research and development in the area of antithrombotic drugs with the development of a variety of new agents. Dr Gray, who is primarily responsible for this area has only one scientific officer, and has had to cope with a large increase in control work. It is requested that consideration be given to the creation of an additional scientific officer post in this area to facilitate the research and development programme.

4. ACCOMMODATION NEEDS

It was recognised at the time of the last Review that the Division's accommodation was crowded and allowed no room for expansion. Since then we have had to make

space for 3 additional staff to expand the Transfusion Medicine area; although Dr Ouwehand is only at NIBSC for half of his time, he still requires bench and writing space. Currently the situation has eased because of vacancies, but when fully staffed the Division is extremely overcrowded. In a recent survey of the Institute's accommodation, it was found that the working area available per person (scientists and support staff) in the Division was only 50% of that recommended by the MRC. The situation is exacerbated by the need to accommodate visiting workers and grant supported staff, and indeed the current lack of space is a barrier to some of the Division's scientists who wish to apply for external funding.

The physical difficulties of finding additional accommodation are recognised, but it is requested that consideration be given to re-siting some or all of the CSSA facilities, which are adjacent to the Division's laboratories. This would allow the creation of at least 2 additional laboratory units, which would help to relieve the current overcrowding situation. In the more immediate future I have suggested that the room shared with Endocrinology Division could be converted into a cell biology laboratory with access from both Divisions.

5. EQUIPMENT NEEDS

The Division is well equipped for the basic techniques of coagulation assays and enzyme kinetics, and equipment for protein purification and molecular biology is adequate. Our cell biology facilities are adequate for current work but leave no room for expansion. Dr Gaffney and Dr Longstaff will both require increasing use of these facilities as studies of fibrinolysis on cell surfaces expand and it may be necessary to consider purchase of an additional flow cabinet and incubator; this would depend on accommodation changes since the existing cell biology laboratory is not large enough for additional equipment.

Many of the more research-based studies in the Division involve detailed measurements of biologically important interactions, eg ligand binding, antigen-antibody etc. A new method of measuring these interactions, called "biospecific interaction analysis" (BIA) was recently developed by Pharmacia Ltd and has been adopted by many Pharmaceutical Companies and research groups. Two machines, the "BIAcore" and "BIAlite" have been developed by Pharmacia, and in a recent trial in the Division, gave excellent results in measurement of antigen-antibody interaction. The possible applications of one of these machines are summarised as follows:

Potential Uses of BIAcore in Haematology

BIAcore is a recently developed technology designed to measure the binding of macromolecules. Observations are readily translatable into association and dissociation rate constants, and affinity constants, and this provides information on biological activities of proteins, carbohydrates or nucleic acids, not so readily, or rapidly, investigated using other approaches. Most areas of work in the Division of Haematology could benefit if this technology was available, including blood products, antithrombotic drugs, fibrinolysis and transfusion medicine. Some specific examples of applications are outlined below:

BIAcore has already been used, in work with Dr T W Barrowcliffe, Dr C Longstaff and Ms J Watton in collaboration with other groups, to look at the binding of AT to a monoclonal antibody with affinity for the inhibitor's heparin binding site. This work demonstrated the ability of this technology to determine rapidly association and dissociation rate constants and a dissociation constant for this interaction. The effects on binding of different heparins to mutant AT molecules and partially denatured AT preparations were easily investigated without any need for labelling as required in the current IRMA assay. Thus the value of this approach in investigating antithrombotic drugs has already been demonstrated.

Dr G Kemball-Cook has also been involved in a collaboration to compare the binding of wild type and mutant FVII molecules to tissue factor using BIAcore technology. It is envisaged that this approach could be readily applied to other coagulation proteins including FVIII, FIX, FX, thrombin and protein C. Phospholipids could also be used and activation complexes built up on the surface of the BIAcore sensor chip. In terms of control work binding properties of wild type and variant forms of recombinant FVIII, and interactions with anti-FVIII mabs could be examined.

Dr W Ouwehand has also identified several areas of work in transfusion medicine in which BIAcore could be applied. These involve recombinant antibody fragments against plasma and recombinant FVIII, and against other platelet membrane glycoproteins currently under investigation in the Division.

In a related field Dr S Thorpe has also identified the characterisation of anti-D antibodies as an area well suited to investigation by BIAcore. Anti-D from several manufacturers is currently under development or clinical trials but there is a need to establish how differences in glycosylation patterns produced by different cell lines influence rates of binding and overall affinity.

In the area of fibrinolysis, some work is planned by Dr C Longstaff and Ms R E Merton in collaboration with a Swedish group who have already used this technology to study tPA-PAI-1. Site directed mutagenesis has been used to generate a range of tPA mutants, proteins with deletions in the active site catalytic triad, which are virtually inactive. With these mutants inhibitor binding could not be followed by monitoring enzyme activity and BIAcore provides an ideal methodology to investigate binding of PAI-1 to these modified tPA enzymes.

Other Applications in NIBSC

The BIAcore system has been used in many biological areas, including HIV research, endocrinology, immunology and bacteriology and is likely to be of interest to several Divisions.

Cost

The fully automated BIAcore machine is £100 000. The cheaper manual version, the BIAlite is £50 000.