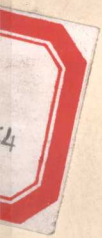
Progress in Transfusion Medicine

Volume 1

Edited by

John D. Cash



CHURCHILL LIVINGSTONE
Medical Division of Longman Group Limited

Distributed in the United States of America by Churchill Livingstone Inc., 1560 Broadway, New York, N.Y. 10036, and by associated companies, branches and representatives throughout the world.

First published 1986

ISBN 0 443 03261 0

British Library Cataloguing in Publications Data

Progress in transfusion medicine. — Vol. 1 –
1. Blood — Transfusion
615'.65 RM171

Printed in Great Britain by
Butler & Tanner Ltd, Frome and London

Preface

Over the last five years there has been increasing concern, worldwide, that the integration of blood transfusion services into local and national health care services is less than satisfactory. A committee of experts of the Council of Europe came to the same conclusion and noted that in many countries there is little evidence of co-ordination, but much evidence of fragmentation of effort and inadequate clinical monitoring of the use of costly and scarce human blood resources. These could lead to inappropriate treatment of individual patients with attendant waste and possible health risks. The committee recommended that there was an urgent need for the creation of national and community based blood transfusion centres which should be centres of excellence in a newly created specialty, transfusion medicine, and made specific proposals with regard to the training of doctors who would work in such centres.

Colleagues in the United States of America have shared these concerns and the National Institute of Health is currently investing significant resources into developing a Transfusion Medicine Academic Award Programme.

The arrival of a new annual publication entitled *Progress in Transfusion Medicine* is a direct result of this rapidly growing worldwide interest. Whilst the views of individual chapter authors may not necessarily be shared by the editor, the topics covered will mirror the extensive curriculum proposed by the European committee of experts. It can be anticipated that particular, but not exclusive, attention will be placed upon the clinical aspects of transfusion practice.

Edinburgh, 1986 J.D.C.

Contributors

Jean-Pierre Allain MD
Centre National de Transfusion Sanguine, Les Ulis, France

John A. J. Barbara MA MSc PhD
Top Grade Scientific Officer; Head of Microbiology Department, North London Blood Transfusion Centre, Edgware, Middlesex, UK

Robert M. Barr MD FRCP(C)
Medical Director, London Centre Canadian Red Cross Blood Transfusion Service, London, Ontario; Director, Blood Bank and Chief, Hematology Service, University Hospital, London, Ontario, Canada

Colin H. Cameron MA MB BChir FRCPath
Senior Lecturer, Department of Virology, Middlesex Hospital Medical School, London; Honorary Consultant, North London Blood Transfusion Centre and Blood Products Laboratory, London, UK

Jan W. ten Cate MD PhD
Senior Lecturer, Division of Haemostasis and Thrombosis, Faculty of Medicine, University of Amsterdam, The Netherlands

Claes F. Hogman MD DMSci
Associate Professor of Immunolgy and Director, Blood Centre, University Hospital, S-751 85 Uppsala, Sweden

Douglas W. Huestis MD
Professor of Pathology, Chief, Immunohematology, University of Arizona, College of Medicine, Tucson, Arizona, USA

E. S. Lennox BS PhD
Director of Immunology, Celltech, Slough, UK

David Brian Lorimer McClelland BSc PhD FRCP(Edin)
Director, Edinburgh and South East Scotland Regional Blood Transfusion Service, Royal Infirmary, Edinburgh, UK

Ruthven Mitchell BSc(Hons) MB ChB MD FRCPath FRCP(G)
Regional Director, Regional Blood Transfusion Centre, Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, UK

R. A. Perrault
London Centre Canadian Red Cross Blood Transfusion Service; London, Ontario, Canada

E. Angela E. Robinson MB BS MRCPath
Consultant Haematologist, Yorkshire Regional Blood Transfusion Service, Regional Transfusion Centre, Leeds; Honorary Consultant Haematologist, Seacroft Hospital, Leeds, UK

Douglas Voak BSc PhD MRCPath
Top Grade Scientific Officer, Regional Transfusion and Immunohaematology Centre, Cambridge, UK

P. L. Yap BSc(Hons) MB ChB PhD MRCPath
Consultant in Blood Transfusion and Immunology, Edinburgh and South East Scotland Regional Blood Transfusion Service, Royal Infirmary, Edinburgh, UK

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Monoclonal antibodies for laboratory aspects of transfusion practice

INTRODUCTION

Safe blood transfusion practice requires bulk supplies of three reagents: those for ABO grouping, Rh(D) typing and antiglobulin reagents for antibody detection and identification.

ABO and Rh(D) antisera from human donors are polyclonal antibodies and tend to be expensive and variable in quality, partly due to the high workload involved in processing large numbers of separate and small donations. This is further exacerbated if these reagents are not obtained from selected and hyperimmunised donors. Monoclonal antibodies can be produced, ex vivo, in unlimited quantities and also offer considerable advantages in terms of sustained high quality.

Monoclonal antibodies to many red cell antigens other than ABO and Rh(D) have now been reported and recently reviewed (Voak and Tills, 1983), but these are beyond the scope of this discussion. The intention in this chapter is to discuss the current and likely future impact of monoclonal antibodies in those areas of transfusion practice that require bulk supplies of reagents.

THE PRINCIPLES OF PRODUCING IMMORTAL ANTIBODY-SECRETING HYBRID-MYELOMA CELL LINES

Conventional sera are mixtures of many antibodies, each made by the progeny of a single cell. The progeny of this cell constitutes a clone of cells and each member of the clone makes the same pure or monoclonal antibody. Unfortunately, antibody-forming cells from spleen or lymph nodes cannot be grown in culture — they die within days.

Kohler and Milstein 1975 reported the successful fusion of normal mouse antibody-secreting lymphocytes with the cells of a myeloma cell line, thus producing a hybrid-myeloma which combined the properties of cell growth and antibody secretion. The hybrid cell could be grown in tissue culture and yield useful antibody in the supernatants.

The sequence of events in the production of monoclonal antibodies has been described in detail (Galfre and Milstein, 1981; Voak and Lennox, 1983) and is briefly summarised below and in Figure 1.

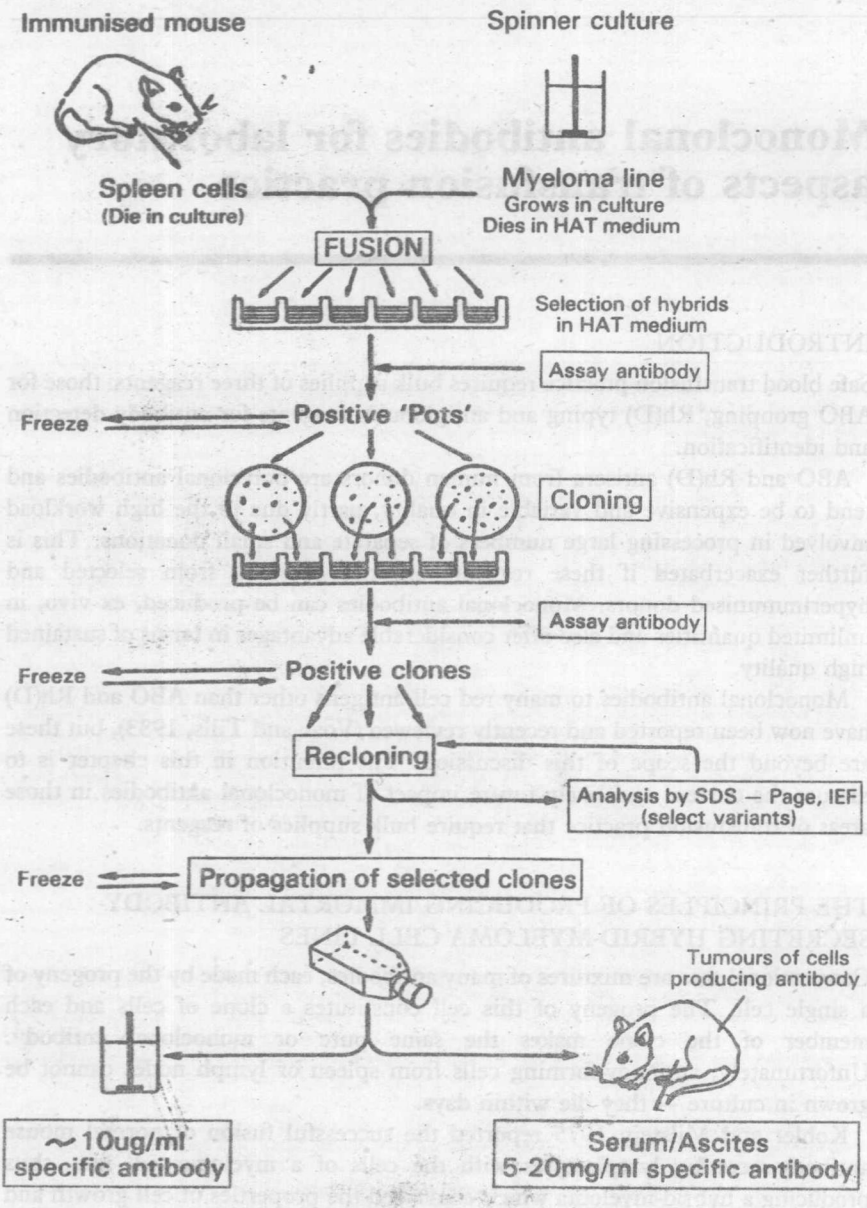

Fig.1.1 Sequence of events in the production of monoclonal antibodies. (Reprinted with permission of Academic Press, New York; Galfre and Milstein, 1981.)

1. Immunisation of a suitable animal.
2. Fusion of spleen cells from the immunised animals with suitable myeloma cells using polyethylene glycol.
3. Selective growth by means of HAT medium (Littlefield, 1976), which contains Aminopterin to kill of the myeloma cells which would otherwise overgrow the hybrid-myeloma cells.
4. Selection of the useful antibody-secreting cell lines by simple screening tests on supernatants.
5. Purification of the cell lines by cloning at least twice.
6. Storage of stocks of the cloned cell line in liquid nitrogen to guarantee quality and supply of the cell line.
7. Determination of the antibody heavy and light chain composition by autoradiograph study of the SDS-PAGE analysed antibody from cell cultures grown in medium containing C14 lysine. It is important to know whether there is contaminating light chain from the myeloma parent cell because early experience has shown that immunoglobulin containing this light chain results in inefficient agglutinating antibodies (Voak et al, 1980). This problem has been overcome in our laboratory using a superior myeloma cell line (NSO) which does not produce light or heavy chains (Galfre and Milstein, 1981); thus all antibodies produces from the fusion products of this myeloma are of the HL type, i.e. all immunoglobulin molecules will be of spleen cell origin.

Figure 1.2 shows two examples of HLK antibodies, the first mouse monoclonal anti-A W6/1 (Barnstable et al, 1978) is an IgM HLK antibody and the rat monoclonal anti-C3g (complement C3) YB2/9 is an IgG HLK

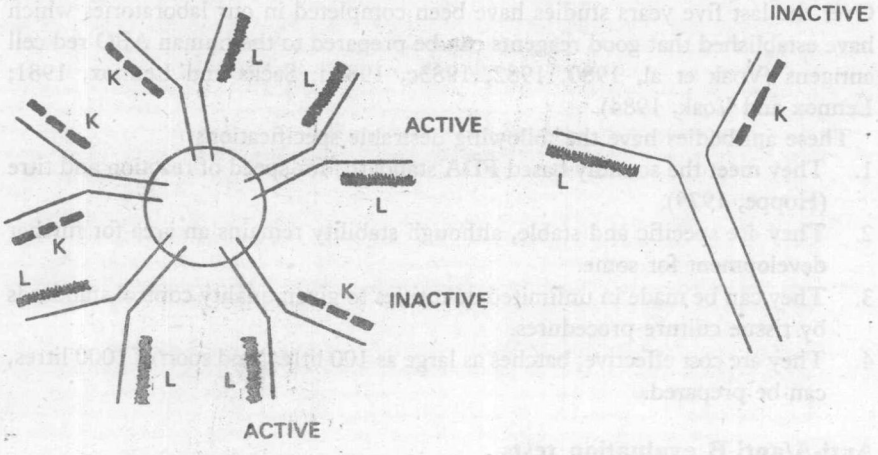

Fig. 1.2 HLK antibodies: a mixture of K myeloma and L spleen light chains.

antibody. HLK hybrid myelomas (Voak et al, 1984) make a variable mixture of three types of molecules, (HL, HLK and inactive antibody HK). HL cell lines can be obtained by screening many sub-clones of the HLK cell line, and this can be a useful way of rescuing useful antibodies rather than starting with new fusions (Voak et al, 1984, 1985b).

8. Antibody production by the selected hybrid myeloma cell line can be achieved by either growing it in tissue culture or injecting it into the peritoneal cavity of mice to produce ascitic fluid. The ascitic fluid approach produces higher concentrations of antibody, but we prefer the use of tissue culture for three reasons:
   a. Unlimited and reproducible quantities of unvarying antibody in tissue culture are produced whereas ascitic fluid antibody can vary from animal to animal both in concentration and degree of contamination with unwanted antibodies. Pooling batches of ascitic fluids will minimise variations, and laboratories lacking bulk tissue culture facilities or expertise may find it wholly acceptable.
   b. The stability of culture supernatant antibody may be superior to that from ascitic fluid.
   c. The use of tissue culture for routine reagent production is ethically preferable.

MONOCLONAL ANTIBODIES FOR USE IN ROUTINE BLOOD TRANSFUSION PRACTICE

ABO antibodies

Over the last five years studies have been completed in our laboratories which have established that good reagents can be prepared to the human ABO red cell antigens (Voak et al, 1980, 1982, 1983c, 1983d; Sacks and Lennox, 1981; Lennox and Voak, 1984).

These antibodies have the following desirable specifications:

1. They meet the soundly-based FDA standards for speed of reaction and titre (Hoppe, 1979).
2. They are specific and stable, although stability remains an area for further development for some.
3. They can be made in unlimited quantities to given quality control standards by tissue culture procedures.
4. They are cost effective; batches as large as 100 litres, and soon of 1000 litres, can be prepared.

Anti-A/anti-B evaluation tests

Avidity and potency

The data in Tables 1.1–1.4 compare the activity of some monoclonal anti-A and anti-B reagents against conventional (non-monoclonal) reagents. The first of our anti-A products (6D4) to be evaluated (Voak et al, 1980) was a significant

Table 1.1 Avidity (potency) tests showing the development of monoclonal anti-A with supernatant anti-A monoclonal antibodies and control reagents

	UK slide tests with 20% A_2B cells in saline		
Anti-A	Avidity (a)	Agglutination at 2 min	Agglutination at 5 min
BGRL[a] 8132 (UK serum)	38	+	+/++
Commercial X[b] (USA)	10	+/++	++/+++
6D4 (conc. x 3)	15	+	+/++
3D3 neat	9	++	++/+++
MHO4 neat	4	+++	++++

	FDA slide tests with 35% cells in own plasma[c]			
	Avidity (s)			
Anti-A	A_1	A_2	A_1B	A_2B
Commercial X (USA)	2	3	2	5
1st 6D4 (x 3)	2	3	2	6
2nd 3D3 neat	2	3	3	4
3rd MHO4 neat	1	1	1	2

[a] BGRL: Blood Group Reference Laboratory, UK.
[b] Commercial X: a commercial conventional hyperimmune anti-A reagent that meets FDA requirements.
[c] After 2 min all gave agglutination >1 mm

Table 1.2 Avidity (potency) tests with supernatant anti-B monoclonal antibodies and control reagents

	UK slide tests with 20% A_1B cells in saline		
Anti-B	Avidity (a)	Agglutination at 2 min	Agglutination at 5 min
BGRL 8835 (UK serum)	6	+++	++++
Commercial X (USA)	4	++++	++++
NB1/19 neat	7	+++/++++	++++
5A5 neat	6	++++	++++
5B2 neat	9	+	+++
3B4 neat	4	+++	++++

	FDA slide tests with 35% cells in own plasma[a]		
	Avidity (s)		
Anti-B	A_1B	A_2B	B
BGRL 8835 (UK)	7	6	5
Commercial X (USA)	4	3	3
NB1/19 neat	4	4	4
5A5 neat	3	3	3
5B2 neat	4	3	4
3B4	5	4	3

[a] after 2 min all gave agglutination >1 mm except 5B2: see text.

improvement over the then locally prepared polyclonal products, but was inferior to commercially available FDA standard reagents as shown by reactions with selected weak A_2B red cells (table 1.3). Our second monoclonal anti-A (3D3), however, equalled many commercial hyperimmune anti-A reagents (Lowe et al, 1984) and a third generation mouse monoclonal anti-A (MHO4) is

Table 1.3 Saline titres of tissue culture supernatant monoclonal anti-A reagents

2% cells Saline RT	6D4 conc. x 3	3D3 neat	MHO4 neat	Group B Commercial X (USA)
A_1	512	1024	1024	512
A_2	256	512	512	256
A_1B	512	512	512	512
A_2B	64	64	256	64
A_2B (weak)	4	16	256	32
A_3B	1	4	256	8
A_3	4	4	256	1
A_x	0	0	64	0

Saline tests negative against A_m, B and O cells.

not only superior to good hyperimmune (polyclonal) anti-A reagents, especially in tests with weak A_2B and A_3 cells, but also detects most examples of A_x red cells better than anti-A,B reagents (Table 1.4) (Voak et al, 1985). Similar progress has been reported from France (Salmon et al, 1983; Lee et al, personal communication) and Sweden (Messeter et al, 1983, 1984).

Table 1.4 Saline titres of tissue culture supernatant monoclonal anti-B reagents

2% cells Saline RT	NB1/19	3B4	5A5	5B2	Group A serum Commercial X (USA)
A_1B	256	64	256	128	64
A_2B	512	128	512	256	64
B	512	128	512	256	128
B cord	256	64	256	128	64
B weak	0	32	64	32	64
A_1 B weak	0	16	64	16	64
A_1 acq.B (Ho^a)	0	0	0	0	4

Saline tests negative against A_1 and 0 cells.

[a]Supplied by Mrs P. Leake, South London BTS.

The original anti-B line NB1/19 (Sacks and Lennox, 1981) has been superseded by a second generation 5A5, 5B2 and 3B4 which are more stable and detect weak B variant blood types missed by NB1/19 (Table 1.4) (Voak et al, 1983c).

In assessing the avidity and potency of these monoclonal antibodies there are certain features which are worth emphasising. The time in seconds to produce macroscopic agglutination after mixing one volume of antibody with one volume of indicator cells is a rapid means of evaluating the avidity of reagents. Avidity tests in saline are to be preferred for demonstrating qualitative differences between reagents because the presence of serum or plasma proteins enhances the agglutination reactions and reduces the avidity time differences seen between the various reagents. However, the serum potentiated slide procedure is the best slide ABO grouping method with known potent reagents, although many prefer spin tube methods for rapid reliable ABO grouping tests.

The use of strong indicator cells may fail to demonstrate an inadequate reagent (Voak et al, 1980, 1982, 1983c). Thus for anti-A evaluation weak A_2B red cells

and not strong 'A_2B' A_1 or A_2 cells are essential. Similarly A_1B cells with a lower B status than B or A_2B cells should be used to evaluate anti-B reagents.

Anti-A/anti-B antibodies for slide test use must produce large clumps of agglutination comparable to those of hyperimmune commercial reagents with the weakest types of A or B red cell types. Anti-B 5B2 is not satisfactory for slide ABO grouping but is still an excellent reagent for tube tests and indeed will detect weak B variant red cells that are not detected by reagents which are superior to 5B2 in the slide test.

Potency tests on monoclonal antibodies are best done with a saline diluent containing 3 per cent bovine albumin. This prevents the sticking of agglutinates to tubes which can lead to incorrect measurement of potency, which is seen with a saline-only diluent. We would also recommend the use of a 5 per cent red cell suspension, rather than 2–3 per cent. Stickiness is not a problem with neat reagents for routine practice as the reagents provided contain foetal calf serum (2–5 per cent).

Stability

The most useful test in our experience is the accelerated stability test of one month at 37°C. Antibodies stable in this test are also stable at six months at 4°C and three months at 25°C, as recommended by the WHO.

Routine tests with monoclonal anti-A and anti-B

The IgM anti-A/anti-B antibodies are potent haemolysins in the presence of serum complement. For routine testing these reagents are therefore formulated to prevent haemolytic reactions by the addition of EDTA (0.02 mol/l) at pH 7.1–7.3. Some of these antibodies show decreased potency at low pH (below pH 5.0) against weak subtypes, but are otherwise stable over a range of pH values (Voak et al, 1983c).

The monoclonal ABO grouping reagents, anti-A (3D3) and anti-B (NB1/19) have now been extensively tested in UK Regional Transfusion Centres and Hospital blood banks, using both manual and automated ABO blood typing tests, and proved to be satisfactory (Voak et al, 1983c; Lowe et al, 1984). Over 250 000 donor blood samples have been tested on the Technicon Autogrouper 16c machine at the Cambridge Regional Transfusion Centre. Here the reagents are enhanced by bromelin and methyl cellulose. Extensive studies would indicate that these reagents will be economic, for 1 litre of each reagent used (at a 1+9 dilution) was sufficient to group 64 000 donor samples on the 16c machine (Voak et al, 1982).

The most difficult routine ABO grouping test is the detection of weak A_2B ('A_3B') red cells for they give a high proportion of free cells in haemagglutination tests with most anti-A reagents. Tests on the Technicon 16c machine, which is designed to interpret reactions based on a 20 per cent free cell baseline, are further complicated by inter-channel variations (Hurd et al, 1984) and the anti-A (3D3) has given false negatives (4 out of 440 AB samples) in typing 64 000 donor

samples. In routine practice this does not cause difficulties because the tests with the donor plasma against group A_1 and A_2 red cells do not match and as a consequence manual check grouping is required. This type of weak A_2B is detected in *manual tests* by the same monoclonal anti-A (3D3), which gives comparable titres to those of conventional hyperimmune reagents (Voak et al, 1983c; Lowe et al, 1984). This problem is likely to become historical because a new monoclonal anti-A (MHO4) gives strong agglutination with the difficult A_2B ('A_3B') red cells and reliably detects them on the 16c machine.

Anti-A (MH04) and the new anti-Bs (5B2 and 5A5) are all excellent machine reagents and they give reliable results at higher dilutions (around 1/50) than the already very economic 1/10 dilutions of anti-A (3D3) and anti-B (NB1/19).

Anti-A (3D3), anti-B (NB1/19) and the new anti-A (MHO4) and anti-B (5A5) perform well in spin tube, sedimentation tube, slide tests and microtitre plate tests. The new anti-B (5B2) is satisfactory in all but slide tests. Certain blends of different anti-B monoclonals show improved speed of agglutination and clump size especially in FDA slide tests, whereas for tube tests we have found that the best anti-B (5A5) is not enhanced by blends with other anti-B monoclonals.

Our experience has shown that monoclonal anti-A and anti-B do not miss strong A or B red cell types. The advantage of the newer reagents is limited to the detection of weaker subgroups. With the exception of slide tests, use of the best single monoclonal anti-A or anti-B is the most cost-effective approach to adopt in reagent production. We would therefore suggest that any proposals for blending monoclonals for reagent production should be based on demonstrated practical advantages and not on theoretical considerations of possible different epitopes of the red cells and avidities of the antibodies.

A1/A2 subtyping

A monoclonal IgG anti-A (3D1) can be used for subtyping (Voak et al, 1983c) and the results are comparable to those obtained with Dolichos biflorus standardised as an anti-A_1 lectin (Bird, 1952). IgG anti-A, like the small molecular sized Dolichos lectin, reacts far more strongly with A_1 cells than A_2 because of the difference in A site density, 1 000 000 vs. 250 000 sites respectively (Williams and Voak, 1972).

Anti-A + anti-B and anti-A,B reagents

Suitable potent conventional group O sera are used as a check reagent to confirm the anti-A and anti-B reagent tests. They are also used, especially in the UK, for the detection of weak A/B blood groups (A_x and stronger A_m types and B_w, B_x etc.) which are not usually agglutinated by anti-A or anti-B reagents.

Monoclonal anti-A,B reagents have now been described, but no single one can fulfil the required function as the anti-A,B antibodies have a preference for A or B and therefore are deficient for either weak A or weak B antigens (Voak et al, 1983c). Thus 'O serum type' reagents must be made by a blend of at least two monoclonal antibodies to meet all the criteria expected of them (see Table 1.5).

A monoclonal anti-A,B (ES.15) developed in Edinburgh (Moore et al, 1983, 1984) reacts well with all A bloods and, when blended with the new anti-B (5B2), also reacts well with weak B bloods, and is a satisfactory anti-A,B + anti-B reagent for routine use. Messeter et al (1984) and Lee (personal communication) have also found good crossreacting anti-A,B antibodies useful in blends to make O serum type reagents.

The introduction of anti-A (MHO4) which agglutinates A_x cells is likely to eliminate this problem for it is the first anti-A that can be blended with a suitable anti-B to make an anti-A + anti-B reagent that will detect weak subgroups of both A and B (Table 1.5).

Table 1.5 Monoclonal reagents agglutinating both A and B cells that may be used instead of group O serum reagents

	Blends			
Anti-A,B ES.15	Anti-A,B ES.15 + Anti-B 5B2	Anti-A MH04 + Anti-B 5B2	Conventional BGRL 1837 (UK) 0 serum	
				2% cells
V	V	C	V	A_1
V	V	C	V	A_2
(+)	V	C	V	B
V	V	C	V	A_2B
+	+	+	+	A_3
+ +	+ +	V	+ +	A_3 (5439)
+	+	(+)	+ +	A_x
+/+ +	+/+ +	+ +	+ +	A_x
–	+ +	+ +	+	B_w
–	–	–	–	0 cells

Absorption, elution and inhibition tests

Absorption and elution tests

Selected monoclonal anti-A/anti-B reagents can be used in absorption/heat elution studies for the demonstration of A or B antigens on weak A or B subtypes not agglutinated by anti-A/anti-B reagents. The anti-A (6D4) does not react with A_x cells (Voak et al, 1980). Anti-A (3D3) behaves like conventional anti-As in not agglutinating, but absorbs and elutes from A_x cells (Lowe et al, 1984), while the latest anti-A (MHO4) agglutinates most A_x cells and thus renders obsolete the need for absorption and elution in most cases, especially if used with papainised cell tests that enhance the reactions.

Inhibition tests

Conventional anti-A/anti-B reagents contain far less antibody than potent monoclonal anti-A/anti-B reagents. Thus these reagents need to be considerably more diluted than conventional reagents for inhibition tests, using varying dilutions of salivas against fixed volumes of antibody, e.g. 1/50 for a monoclonal antibody compared to 1/5-1/10 for conventional reagents.

Freedom from false-positive reactions

As long as care is taken to ensure that the foetal calf serum used in growth medium is antibody free, then it can be certain that the monoclonal reagent will not contain additional antibodies. Hence monoclonal antibody reagents are free of false positives. They do not react with T active or acquired B red cells and are useful in difficult cases in transfusion practice or forensic serology (Voak et al, 1983b).

MONOCLONAL ANTI-D ANTIBODIES

Unfortunately, the rodent hybrid-myeloma system has been unable to produce anti-D-like antibodies that are suitable for transfusion practice. However, specific monoclonal anti-D has been produced from cultures of Epstein Barr Virus (EBV) transformed human anti-D-secreting B cells prepared from the buffy coats of hyperimmunised anti-D plasmapheresis donors.

EBV derived anti-D

EBV is a lymphotropic herpes virus and human B-lymphocytes have a receptor for it. Because T cells do not have a receptor for EBV, and T cells may kill EBV infected B cells, it is essential to remove T cells from the B cell preparation before attempting EBV treatment of the B cells. This is achieved by rosetting the T cells with amino-ethyl-thio uronium bromide (AET) treated sheep red cells (Boylston et al, 1980; Koskimies, 1980). Infection of B cells with EBV transforms them to give cell lines which grow in tissue culture and produce antibody.

The pioneer work in 1980 of Koskimies and Boylston et al resulted in the production of polyclonal IgG and IgM anti-D antibodies, respectively. However, these uncloned EBV transformed cells were unstable and only survived in tissue culture for up to three months. Both groups sought to clone the cell lines but were not successful. However, in 1983 Crawford et al (1983b) reported success. This was achieved by improving the rosette selection of the anti-D-secreting cells, using the very rare -D- cells that have 15 times as many D antigen sites per cell than normal D positive cells, combined with papain enzyme treatment of the -D- cells to further increase antibody cell reactions; using irradiated feeder cells with low numbers of EBV infected anti-D-secreting cells; and early cloning of the cell line. Doyle et al (1984) have further developed the Crawford procedure, paying particular attention to minimising mycoplasma infection, which they claim is one of the main causes of failure with EBV cell lines. They have produced many cloned EBV anti-D-secreting cell lines.

Most workers have found that the EBV anti-D cell lines are difficult to stabilise and grow in bulk culture, and up to now only the D4 cell line of Crawford et al (1983a) has grown well in bulk culture. Recently the Stanford

group have fused an EBV anti-D cell line to a human-mouse hetero-myeloma (SHM-D33) that is a suggested substitute for a human myeloma line. They have obtained two IgG3 anti-D-secreting clones (D4.B2 and E10-C1) which have been stable for over eight months and which may be capable of bulk anti-D production (Bron et al, 1984).

Agglutination studies

The examples shown in Table 1.6 show that tissue culture can provide IgG anti-D antibody titres that are more than adequate for reagent use by albumin enhancement tests.

Table 1.6 Agglutination studies on EBV monoclonal IgG anti-D antibodies

	Albumin displacement tube tests at 37°C × R_1 r 2% cells									
Cell line	Dilutions of supernatants[a]									
(Source)	1	2	4	8	16	32	64	128	256	iu/ml
(Doyle & Bradley)										
CC2	C	C	C	V	V	V	+	(+)	W	23.3
DC2	C	C	C	C	C	V	+	(+)	O	25.5
EC3	C	C	C	V	V	V	+	+	W	26.0
FB6	C	C	C	C	C	V	+	(+)	O	22.1
(Crawford)										
D4	V	V	V	V	++	+	+	+	GW	16.1

[a] All negative x panels of D negative cells.

Initial trials by Crawford et al (1983a), Cambridge, Bristol and Stanford (USA) workers have demonstrated the suitability of these anti-Ds for routine reagent development, although individual antibodies miss some D_u and D variant bloods. The antibodies work well with papain (enzyme) treated cells (washed to remove the papain) and in antiglobulin reagent tests. These IgG anti-D molecules can also be modified by a mercaptan, e.g. DTT, which increases their agglutinating efficiency so that they agglutinate in saline tests. However, in enzyme tests using 'in line' papain and bromelin solutions the antibodies are destroyed, even those of the IgG 1 subclass because the tissue culture supernatant contains less protein than human serum and there is thus excess enzyme.

It seems certain that trials of blends of several monoclonal anti-Ds will begin in the near future in order to assess their reliability for the detection of D_u and D variant bloods: the most difficult part of RhD red cell typing work.

Safety aspects

EB virus has been categorised as a C pathogen (Howie, 1978). The current view is that these reagents are safe for routine laboratory use after they have been suitably filtered (0.1 μm) to remove residual active EB virus (Crawford et al, 1983b).