Immune-mediated cell separation methods
Philip A. Dyer
North Western Regional Tissue Typing Laboratory, St Mary's Hospital, Hathersage Road, Manchester M13 0JH, U.K.

Introduction
The diversity among cells of the body can be revealed in vitro by physical methods such as light- and electron-microscopy, size and density separations, active and physical adhesion to a solid phase and electrophoretic methods. Recently, much progress has been made in our understanding of the functions of cells by using antigen/antibody interactions to perform negative and positive selections for isolation of discrete populations and sub-populations. Such immune-mediated techniques heavily exploit the specificity of monoclonal antibody technology and benefit from the use of biological probes.

Usually, probes are antibodies, often monoclonal, directed specifically to cellular antigens such as cell-membrane proteins. Three main approaches can be identified: (a) antibodies to cell-surface phenotypic markers are used to identify cell populations in situ such as in immunohistology, flow cytometry and rosetting, (b) antibody-mediated cytotoxicity which is usually a negative selection of a viable cell population and (c) antibody linked to a solid phase which can in turn be selected by techniques such as centrifugation (Staphylococcus aureus), column chromatography (Sepharose) or magnetism (Dynabeads, Magnebeads).

These techniques are subject to usual technical problems such as contamination, variation in viability of the cells isolated and yield, so careful monitoring of the isolate and control of the procedures is essential. When using samples from 'normal' individuals regard must be paid to age and state of health since cell profiles vary. In patients, even more care is needed since disease is manifested by changes in tissues. In instances such as leukaemias, cell populations shift dramatically and may be severely reduced (e.g. acute myeloid leukaemia) or present in greatly increased numbers (e.g. chronic lymphocytic leukaemia).

The application of immune-mediated cell-separation techniques in medical diagnostics and therapy varies from diagnosis of tumours by identification of altered antigen expression using immunohistology to preparation of donor bone marrow by removal of mature immunocompetent T lymphocytes. These techniques continue to be a rapidly developing area in management and treatment of patients. This paper describes some of the procedures in established use in clinical laboratories and particular attention is paid to antibody-coated magnetizable microspheres which have great potential for many areas of clinical science.

Cell-surface markers
Collaborative workshops [1] have identified a large number of cell-surface molecules which are termed cluster of differentiation (CD) antigens which can be identified by antibody probes. Many CD antigens are specific to cell types (e.g. CD19 is a marker of B lymphocytes) while others are common to a cell lineage (e.g. CD9, which is found on most T cells, B cells and myeloid cells). Monoclonal antibodies specific for CD antigens are available from the originating laboratory often as a gift for research purposes and sometimes commercially. They are most often used in fluorescence assays coupled with fluorescein isothiocyanate although many other dyes can now be coupled to allow multiple-staining techniques.

Such fluorochrome-tagged antibodies can be attached to cell populations by simply incubating target and probe usually at 4°C for several minutes. It is then possible to separate stained cells visually by fluorescence microscopy, as is used in many immunology laboratories to measure T lymphocyte 'helper' and 'killer' cell ratios (CD4/CD8) which vary in many disease states such as human immunodeficiency virus infection and leukaemias. Physical separation is possible by using a fluorescence-activated cell sorter (FACS) which uses a laser to stimulate the fluorochrome tag and electric fields to divert stained cells into a collection vessel. This technique is addressed separately in this symposium.

When target cells are a constituent part of a tissue rather than a free suspension it is possible to incubate thin (10 μm) frozen sections of tissue with fluorescent-antibody probes. Careful washing is essential to remove non-specific background staining. Under the fluorescent microscope bound antibody is seen against the background tissue morphology, which can be counterstained, allowing

Abbreviations used: CD, cluster of differentiation; HLA, histocompatibility locus antigens; MM, magnetizable microspheres; ACMM, antibody-coated magnetizable microspheres.
precise separation of surface marker positive and negative regions of tissue. This technique can also be used with antibody probes labelled with tags visible in ordinary transmitted light such as the immuno-gold system. Non-fluorochrome systems are usually permanent whereas fluorochromes can rapidly fade or bleach under continuous light stimulation.

Some cell-surface molecules have epitopes which bind specifically, for unknown reasons, to other biological particles such as plant lectins or species-different cells. These phenomena are used extensively in human red cell grouping techniques and in immunological research. One clinical application is of separation of T lymphocytes from a cell suspension by rosetting of these cells with sheep red blood cells. The complex rosettes can be gently pelleted from suspension by centrifugation and the T cells released by mechanical means. T lymphocytes prepared in this way can be quantitated or used in functional assays.

**Cytotoxicity**

The induced killing of cell populations is termed cytotoxicity and can be achieved chemically with poisons, or biologically by exploiting such processes as complement-mediated killing.

Antibody probes tagged with poisons such as ricin or labelled with high-specific-activity radio-isotopes have been used to treat tissue- or organspecific tumours. It is arranged that the carrier antibody binds only to tumour markers which it specifically recognizes and the poison tag is thus concentrated on/in the tumour. In time, tumour cells are separated from normal cells by their death. These techniques are largely experimental and have been pioneered by the Imperial Cancer Research Fund, London.

Cell-cytotoxicity assays are more usually performed in vitro in immunological research and patient testing. A classical cytotoxicity assay is used in laboratories supporting organ transplantation to define cell-surface antigens known to govern recipient rejection responses (tissue types or histocompatibility locus antigens (HLA)). The genetic system coding for HLA antigens is among the most complicated known in man and those keen to read further should consult a review [3], detailed coverage of techniques is also available [4]. In outline, viable target cells such as peripheral blood lymphocytes are incubated with alloantisera directed against one or a group of known HLA specificities for 30 min at 22°C followed by a further incubation of 60 min in the presence of an excess of rabbit serum as a source of complement. The test is interpreted by staining of live cells with acridine orange and killed cells with ethidium bromide; these dyes fluoresce green and red respectively under blue light allowing visual separation of cell populations by microscopy. In this assay cells which express, on their surface, antigens to which an antibody is directed will bind that antibody. Subsequently complement in the rabbit serum binds to receptors on both antibody and cell surface; this complex is unstable and results in disruption of the cell membrane and killing of the cell. Cells which do not express a recognized antigen will remain viable at the end of the assay. There are now microscope-based machines which automate reading and interpretation of these assays.

There are other methods of determining the end-point of a cytotoxic-cell separation assay including release of $^{51}$Cr by killed cells but usually non-radioactive methods are preferred.

**Antibody linked to a solid phase**

Physical linkage of antibody probes to a solid phase gives the advantage of possible separation of subsequent bound targets from non-bound cells. A good example is the Protein A of *Staph. aureus* which binds to the Fc portion of an antibody molecule. The cell + antigen/antibody + *Staph. aureus* complex is large and can be removed by centrifugation. It is also possible to isolate cell-free antigen in this way.

Antibody can also be bound to Sepharose beads via Protein A and subsequently cells can be separated on columns of the Sepharose bead complex. This is a form of column chromatography and allows a flexible approach to cell separation with pure yields and viable cells [5].

Recently there has been parallel development by several concerns of magnetizable microspheres (MM) which can be antibody coated (ACMM). Beads are marketed by several companies including 'Dynabeads' (Dynal Inc., Norway), 'Magnabeads' (Magnetic Developments, Chicago) and Flurobeads (One Lambda Inc., Los Angeles). They are of varying size and uniformity but basic principles are the same. At a recent symposium [6] the application of MM was comprehensively reviewed and many instances of separating cells were covered; some are listed in Table 1.

The application of ACMM to clinical tissue typing has gained the most widespread use and this will be used as an example of the techniques involved. The procedure for isolating target cells is shown in Fig. 1.
**Table I**

**Cell separations with MM**

Abbreviations used: NK, natural killer; LAK, lymphokine-activated killer cells.

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Microsphere</th>
<th>Target cell</th>
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</thead>
<tbody>
<tr>
<td>CD4 &amp; CD8</td>
<td>Magnetite crystals and dynabeads</td>
<td>T and B cells</td>
</tr>
<tr>
<td>HLA class II, CD3</td>
<td>Dynabeads</td>
<td>B and T lymphocytes</td>
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<tr>
<td>Ulex europaeus lectins</td>
<td>Dynabeads</td>
<td>Endothelial cells</td>
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<tr>
<td>Synaptosome surface</td>
<td>Magnagel</td>
<td>Intact nerve endings</td>
</tr>
<tr>
<td>Various</td>
<td>Dynabeads</td>
<td>Micro-organisms and microbial toxins</td>
</tr>
<tr>
<td>Various</td>
<td>Dynabeads</td>
<td>Selection and cloning antigen-specific hybribomas</td>
</tr>
<tr>
<td>Tumour specific</td>
<td>Dynabeads</td>
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<td>CD34</td>
<td>Dynabeads</td>
<td>Stem cells</td>
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<td>CD1a</td>
<td>Dynabeads</td>
<td>Epidermal Langerhans</td>
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<td>CD6</td>
<td>Dynabeads M-280</td>
<td>Mouse embryo carcinoma</td>
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<tr>
<td>CD56</td>
<td>Dynabeads M-450</td>
<td>NK and LAK cells</td>
</tr>
<tr>
<td>CD25</td>
<td>Dynabeads</td>
<td>Activated T cells</td>
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**Fig. 1**

**Use of ACMM in tissue typing**

1. Isolate lymphocytes from anti-coagulated peripheral blood sample by density-gradient centrifugation at SG 1.077 (e.g. Lymphoprep, Nycomed, Norway)
2. Pellet lymphocytes by centrifugation in 4 ml polypropylene tube
3. Mix 15 μl Dynabeads Class I or Class II with lymphocyte cell pellet
4. Incubate on ice for 4 min
5. Add 2 ml phosphate-buffered saline at 4°C (PBS). Mix gently
6. Apply tube and contents to a strong magnet for 3 min
7. Remove unbound cells in suspension using pipette while tube remains on magnet
8. Add 1 ml PBS and wash bound cells gently by rotating tube away from magnet
9. Apply tube to magnet for 2 min
10. Remove washings as in (7)
11. Resuspend cells in required volume for cytotoxic assay

While it is possible to isolate T and B lymphocytes using ACMM starting directly from an anti-coagulated blood sample, use of a lymphocyte preparation has advantages, including a reduction in the number of ACMM needed (cost saving), and in most cases the lymphocyte preparation from patients can be stored in liquid nitrogen. By incubating small volumes of density-gradient-purified lymphocytes with ACMM a further reduction in the use of ACMM is possible.
The efficiency of separation of cells bound to ACMM depends on the strength of the magnet used. It is preferable to use rare earth magnets such as neodymium/iron/boron to attract all MM to the side of the tube. One drawback of these magnets is their potential to cause havoc in a laboratory when so much information is stored on magnetic media!

There is no evidence that ACMM remaining on separated cells interferes with functional assays and for tissue typing no attempt is made to remove them. Reagents are available to remove beads if so desired (Detachabead, Dynal, Norway). One benefit of ACMM-bound lymphocytes in tissue-typing assays is the discovery that incubation times can be reduced by half, probably as a result of modulation of the expression of the target cell surface antigen. This has great benefit as time is short when typing cadaveric organ donors. Complementary to definition of tissue types in organ donors and recipients is direct cross-matching of recipient sera with donor lymphocytes. It was shown in the mid-sixties that if recipient sera induced killing of donor cells in vitro then the transplant was usually rapidly rejected with clotting of the renal blood vessels. This was subsequently shown to be the result of antibodies circulating in the recipient which bound directly to mismatched donor HLA antigens on the kidney causing thrombosis. Several studies comparing ACMM-isolated donor lymphocytes with density-gradient-isolated lymphocytes in the cross-match have failed to show a difference between isolation methods in the efficiency of the assay.

Despite the relatively high cost, most U.K. Tissue Typing laboratories now use ACMM in routine HLA typing and cross-matching tests and the benefits of a reduction in time and advantages over out-dated cell separation methods such as sheep red blood cell rosetting are worthwhile.

All routine assays must be robust to allow their use on a daily basis by a number of laboratory staff. For clinical application reliability is particularly important. We (and others) have now used ACMM for routine tissue typing successfully since their introduction in 1988.

**Conclusion**

Cell separation methods which are mediated by immune reactions have become widespread in use throughout clinical laboratories. Recent developments such as ACMM have opened up many possible applications for such assays and have contributed to existing techniques by updating procedures. There is still great potential for application of ACMM and those involved in developing laboratory assays are encouraged to investigate alternative approaches to their problems by using MM.

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