A review of methods available for the identification of both organ-specific and non-organ-specific autoantibodies

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An increasing number of pathological conditions are being identified in which tissue damage occurs in association with immune activation directed against components of normal tissues or organs, the so-called autoimmune reactions. The tissue damage may be confined to individual organs, e.g. the thyroid gland or groups of related endocrine organs, in which case the autoimmunity is termed organ-specific. In other situations the immunological attack manifests as multi-system involvement, e.g. systemic lupus erythematosus, and is termed non-organ-specific. Organ-specific and non-organ-specific autoimmunity is associated with a wide range of overlapping clinical disorders (Table 1). Either humoral or cellular mechanisms or both may be responsible for the pathology seen in autoimmune diseases. Cellular mechanisms are poorly characterized and their investigation is rarely available except in specialized laboratories. Antibodies with a wide range of tissue specificities have been somewhat better characterized.

Many fundamental issues remain to be resolved with respect to the initiating trigger in autoimmune disease, the relationship of this trigger to the subsequent autoimmune activation, and indeed the resulting disease. There is evidence that some antibodies are pathogenic, for example transfer of maternal immunoglobulin G (IgG) in thyrotoxicosis results in transient neonatal thyrotoxicosis. Many other antibodies cannot be shown to be directly pathogenic although they may serve as a useful diagnostic pointer to the presence of autoimmune disease. The more clinical aspects of autoimmunity will be the subject of a forthcoming Clinical Investigation Standing Committee review.

Traditionally, autoantibodies have been identified by reaction against tissues or tissue organelles, most often subjectively using indirect immunofluorescence (IMF) microscopy. A convenient approach to autoantibody testing by IMF was to use a composite block of rodent tissues, made possible by the cross-reactivity between species shown by the common autoantibodies. This block included liver, stomach, kidney and thyroid and was used for the so-called 'autoimmune screen' to detect a number of both organ-specific and non-organ specific autoantibodies. This screening approach should now be replaced by a more selective and clinically focused approach to autoantibody identification using more specific tissues, or specific antigens as these become characterized. Molecular biochemical techniques are contributing to more precise antigen identification and availability of purified antigen preparations, allowing a wide range of immunochemical methods such as radioimmunoassay, gel precipitation, agglutination, nephelometry and particularly ELISA to be developed. The refinement of the analytical techniques has made autoantibody testing available to an increasing number of routine laboratories, with the potential for standardized, quantifiable, fully automated analyses. Increasingly, for the better characterized antibodies, reference preparations are becoming available to enable uniform calibration of assays. Many of these reference preparations can be obtained from the National Institute for Biological Standards and Controls (NIBSC; see Appendix 3).

Although autoantibody testing is becoming more standardized, the clinical interpretation remains complex and it is recommended that laboratories offering autoantibody testing should have available a medical or clinical scientist with specialist immunology training to support the analytical service.

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TABLE 1. *Spectrum of autoimmune disorders*

Specificity	Organ/tissue	Disease
Organ-specific	Pituitary	Hypophysitis
	Endocrine	
	Thyroid	Hypothyroidism Thyrotoxicosis
	Adrenal	Addison's disease
	Pancreas	Insulin-dependent diabetes mellitus
	Gonads	Premature menopause (rare cause) Male infertility (rare cause)
	Gastrointestinal	
	Stomach	Pernicious anaemia Atrophic gastritis
	Intestine	Coeliac disease
	Liver	Primary biliary cirrhosis Active chronic hepatitis
	Heart	Dressler's syndrome
	Eye	Sympathetic ophthalmia Phacogenic uveitis
	Skin	Pemphigus vulgaris Pemphigoid
	Neuromuscular	Myasthenia gravis Lambert–Eaton syndrome
	Haematological	Haemolytic anaemia Idiopathic thrombocytopenic purpura Idiopathic leucopaenia
	Kidney	Goodpasture's syndrome Crescentic glomerulonephritis
	Systemic vasculitides	Wegener's granulomatosis Polyarteritis nodosa
	Connective tissue	Sjogren's syndrome Rheumatoid arthritis Polydermatomyositis Scleroderma Mixed connective tissue disease Discoid lupus erythematosus Systemic lupus erythematosus Anti-phospholipid syndrome
Non-organ-specific		

A large and increasing number of autoantibodies has been identified. This review will consider the assays available for the most frequently assayed of these. Techniques such as indirect immunofluorescence, particle agglutination, and ELISA are commonly applied in the detection of many of the antibodies and the principles of these methods will be discussed in the next section. The specific aspects of these assays, and techniques used for any individual antibody, will be considered in the section relating to that antibody. Each antibody section will review the preparation and identity of the antigenic source, any relevant reference material available, types of assay and commercial suppliers of reagents together with consideration of quality assurance and performance. Relative costs quoted are based on recommended reagent prices and assume analyses according to manufacturers' instructions and with reasonable batch sizes where calibrants and quality control samples are not disproportionally large compared with sample numbers.

SAMPLES AND STABILITY

All serological autoantibody testing is done using serum samples. Samples should be stored at 4°C until assayed and frozen at below −20°C for any long-term storage after testing. Most autoantibodies are remarkably stable as long as samples do not become contaminated by bacteria. For certain agglutination assays samples may need to be heated at 56°C for 30 min to inactivate complement.

PRINCIPLES OF TECHNIQUES

Autoantibodies present in biological fluids are identified exclusively by immunochemical techniques involving detection of the complex formed between the antibody and a target antigen which is presented in tissues, cells, a crude tissue extract, or in a purified or recombinant form.

In immunochemical reactions, addition of increasing amount of antigen to a constant amount of antibody will result in increasing precipitation which reaches a plateau and then falls off, as first described by Heidelberger and Kendall.[1] The decrease in precipitation at higher antigen concentrations is due to the formation of smaller, more soluble complexes; this gives rise to the prozone effect of antigen excess, and will result in false negative results when it occurs in an immunochemical assay. Adequate dilution of samples is needed for any of the assay systems in which a prozone effect could be encountered as detailed in individual sections.

Indirect immunofluorescence

IMF is the most commonly used test for screening for the presence of autoantibodies. The technique is relatively simple and inexpensive and lends itself to rapid throughput of samples. The process is summarized in Fig. 1. Tissue sections or cell preparations may be prepared in the laboratory or are commercially available. Where tissue sections are prepared in the laboratory this will entail the use of frozen material so a suitable freezing microtome (cryostat) will be required. Where fluorescence microscopy is used for detection of autoantibody staining patterns a darkened laboratory area is required.

Diluted samples and controls are incubated with cell preparations or tissue sections which have been fixed to glass slides. Any autoantibody directed to an exposed cellular antigen will bind to the cells or tissue. Excess, unbound material is washed away using phosphate buffered saline (PBS). Bound human immunoglobulin is detected using an antibody conjugated to a fluorochrome. The conjugated antibody is directed against the Fc portion of human immunoglobulin. The most frequently used reagent is a fluorescein isothiocyanate-conjugate (FITC) which is visualized by fluorescence microscopy. The conjugated antibody can be monospecific, i.e. directed against human IgG only, or polyspecific, i.e. directed against IgG, IgA and IgM. The choice of conjugated antibody will depend on the class of immunoglobulin relevant to the disease state under investigation. The optimum dilution for use must be determined for each new batch of anti-human immunoglobulin by the checkerboard method[2] since it will vary from batch to batch even if obtained from the same source.

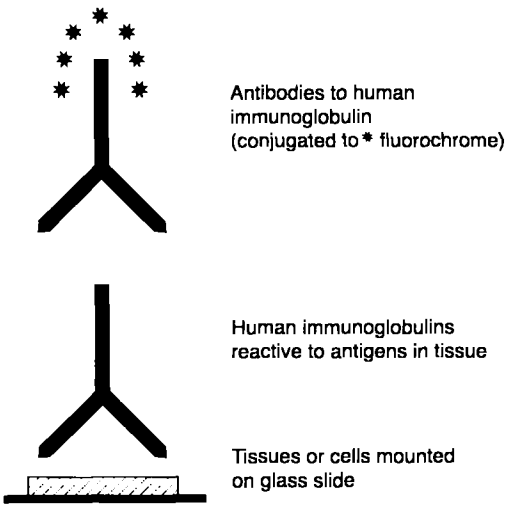

FIGURE 1. *Principle of indirect immunofluorescence. Human serum under investigation is incubated with tissue or cell substrate mounted on a glass slide. Unbound material is washed from the slide leaving only immunoglobulin specifically recognizing antigens in the tissue. Bound human immunoglobulin is detected with an antiserum conjugated to a fluorochrome. The fluorochrome is visualized by fluorescence microscopy.*

Human serum contains antibodies that react with non-human animal tissues but not with human tissue. These antibodies are termed heterophile antibodies and may mimic patterns produced by autoantibodies on the rodent tissues frequently used for screening. These are discussed further in the context of the individual antibodies.

Particle agglutination

Agglutination assays involve the cross-linking of specific antigen-coated particles by an antibody directed against that antigen, to form large macroscopic aggregates. Many of the earliest assays were based on haemagglutination using red blood cells as the antigen carrier. A large number of antibody links was required to overcome electrostatic repulsion. IgM agglutinated better than IgG due to its pentameric structure giving 10 antigen-binding sites per molecule resulting in the charged cells being held spatially further apart.[3] Other particles now used include gelatin and latex,[4] which provide a more inert, stable system. Whilst many of these assays are designed to detect the clinically significant IgG antibodies, low titre cross-reacting IgM antibodies, or classic IgM rheumatoid factors, are a potential source of interference.[5]

Testing is done in the U-shaped wells of agglutination plates so that the pattern can be easily visualized in the bottom of the well. Serial dilutions of samples are made to eliminate problems associated with antigen excess. In the absence of antibodies the particles will settle into a well defined button or ring in the bottom of the well. Agglutination of particles by specific antibodies results in a diffuse layer of particles across the bottom and up the sides of the well. As the sample is diluted the cells become more compact as the antibody is diluted out, and eventually the cells will tend to form a compact button as in the negative antibody control. The titre of the antibody is given by the subjective estimation of the highest dilution demonstrating agglutination. The assays are subject to operator variability in scoring titres and the mathematics of quality control and the significance of differing titres is rarely critically considered.[6]

Enzyme-linked immunosorbent assay (ELISA)

ELISA assays have the antigen preparation coated onto the wells of a microtitre plate. Free binding sites are blocked using an immunologically inert protein. Samples, standards and controls are diluted in buffer, added to the wells and the plate is incubated. Following incubation, unbound protein is removed by washing the plate with buffer. Immunoglobulin bound to the antigen is detected by reaction with an antibody conjugated to a chromogenic enzyme; the conjugated antibody is directed against human immunoglobulin. After washing away unbound conjugate, the reaction is visualized by addition of a corresponding chromogenic substrate and the absorbance measured by spectroscopy. The conjugated antibody can be monospecific for IgG, IgA or IgM depending on which class of immunoglobulin is relevant to the disease state under investigation.

COMMERCIAL SUPPLIERS

In the following sections which consider specific autoantibodies, reagent manufacturers will be referred to in an abbreviated form. The full name of the companies and their addresses and telephone numbers can be found in Appendix 1. We have tried to include all relevant manufacturers; inclusion does not signify any endorsement of the products and we apologise for any exclusions attributable to our ignorance.

RARE ORGAN-SPECIFIC ANTIBODIES

Autoantibodies may be specifically directed against endocrine organs. For a number of these autoantibodies, the target antigen has not yet been characterized so the detection method remains that of relevant IMF patterns using appropriate tissues; the occurrence of some antibodies is rare and their identification is confined to specialized centres with experience in interpretation of patterns and with availability of appropriate control materials. These antibodies include those directed against pituitary, parathyroid, adrenal, islet cells, ovary and testis and as the assays are not widely available they will not be discussed in this review. The limited availability of these assays is reflected in the absence of reference materials and National External Quality Assessment Schemes (NEQAS).

THYROID ANTIBODIES

There are a number of autoantibodies associated with the autoimmune thyroid diseases as shown in Table 2. The most commonly measured and clinically useful autoantibodies will be reviewed in this article. These can be characterized into primary antibodies, which are directly pathogenic and often directed against cell membrane receptors, e.g. thyroid-stimulating hormone (TSH) receptor, and secondary antibodies which do not appear to be involved in the pathogenesis but which can serve as a useful diagnostic marker to the presence of autoimmune disease.

Primary thyroid antibodies

The most well known of these is termed long-acting thyroid stimulator (LATS), which is an

TABLE 2. *Antigen specificities and disease associations of the antibodies involved in autoimmune thyroid disease*

Antigen	Antibody	Disease association*
	Primary	
TSH receptor	Thyrotropin binding-inhibitory Ig	Graves' disease† Hashimoto's thyroiditis Idiopathic hypothyroidism
	Thyroid stimulating Ig	Graves' disease
Unknown antigen	Thyroid growth-stimulating Ig	Graves' disease
	Thyroid growth-inhibiting Ig	Hashimoto's thyroiditis Idiopathic hypothyroidism
Thyroxine Triiodothyronine	Thyroid hormone Ig	
	Secondary	
Thyroglobulin	Thyroglobulin antibody	Hashimoto's thyroiditis Graves' disease Subacute thyroiditis Idiopathic thyroiditis
Thyroid peroxidase	Thyroid microsomal antibody	Hashimoto's thyroiditis† Graves' disease Subacute thyroiditis Idiopathic thyroiditis

*Pathologies listed in order of antibody associated sensitivity and specificity. †High sensitivity (> 80%).
TSH—thyroid-stimulating hormone; Ig—immunoglobulin.

IgG antibody directed against epitope(s) on the extracellular domain of the TSH receptor. The earliest method of detection of this antibody was stimulation *in vivo* of the thyroid of guinea pigs. This autoantibody is now termed thyroid-stimulating immunoglobulin (TSI). Since these earliest assays many others have since been described.[7,8] A number of research and commercial assays exist which show good correlation with the thyroid stimulation assays, and are useful in the diagnosis of Graves' disease specifically. One such method, the radioligand assay, is based on competition between the autoantibody and radiolabelled TSH for the receptor. The antibody detected is referred to as thyrotropin-binding-inhibitory immunoglobulin (TBII). This type of assay has indicated that not all the antibodies detected do in fact stimulate the thyroid, indeed some may be inhibitory. The presence of inhibitory antibody is less sensitive and specific for Graves' disease than the presence of stimulatory antibody activity.[9] The stimulatory and inhibitory effects can only be differentiated by functional assays which measure the generation of cAMP.[10] As such assays are only available at specialized centres they will not be considered further in this review.

There are no recommended methods, accepted standards or quality assurance schemes for these antibodies.

Secondary thyroid antibodies

The major antibodies associated with autoimmune thyroid disease are the thyroglobulin (TG) and thyroid microsomal antibodies, both of which are measured in many laboratories. This is primarily an historical practice since each antibody is associated with distinctive patterns of fluorescence on human thyroid tissue. Although few laboratories still use tissue patterns to identify these antibodies it is most important to use a composite tissue block since anti-mitochondrial antibody demonstrates a similar immunofluorescent staining pattern on thyroid tissue but can be distinguished from thyroid antibodies by reaction with the other tissues, e.g. liver. The few laboratories still using indirect immunofluorescence for the detection of thyroid antibodies use either thyroid sections produced in the laboratory or commercially available primate tissue sections. Indirect immunofluorescence has been largely superseded by agglutination and ELISA assays. The antigens, thyroglobulin and thyroid microsomal antigen, are extracted and partially purified from human thyroid glands. The microsomal antibody is the more clinically relevant and the simultaneous measurement of thyroglobulin antibodies increases the cost without increasing diagnostic effectiveness.[11] The antigen against which the thyroid microsomal antibody is

directed has been identified as thyroid peroxidase (TPO), and is available in a recombinant form for use in sensitive ELISA assays.[12]

Reference material

A reference preparation is now available from NIBSC for both thyroglobulin antibodies (65/93) and thyroid microsomal antibodies (66/387).

Assay types

Agglutination assays

The most frequently used commercial assays use either antigen-sensitized coloured gelatin particles or antigen-sensitized turkey thyrocytes. In these assays uncoated particles or cells are used as an internal control to identify interference from heterophile antibodies. A wide range of possible concentrations of thyroid antibodies are present in normal and disease states, therefore when screening a series of dilutions is used to eliminate problems associated with antigen excess.

ELISA

The recombinant thyroid peroxidase antigen preparation allows minimal cross-reactivity with contaminating thyroglobulin and/or mitochondria found in other microsomal preparations. The antigen is also readily available with little batch to batch variation, thereby avoiding problems associated with variable antigenicity.

Assay characteristics

The agglutination assays are at best semi-quantitative, lack sensitivity (TG ~ 24%, TPO ~ 33%), require experienced interpretative skills, and have been largely superseded by ELISA assays. Compared with immunofluorescence, ELISA assays are as sensitive and specific for thyroglobulin antibodies, although according to the Medical Devices Directorate report,[13] assays for thyroid microsomal antibodies have increased sensitivity at the expense of specificity.

Commercial suppliers report inter-assay coefficients of variation of 5–9% and intra-assay coefficients of variations 8–13% for ELISA assays.

Quality assurance

The UKNEQAS reports both qualitative and quantitative performance characteristics for thyroglobulin and thyroid microsomal antibody, and has recently introduced a distinct grouping for users of the microsomal antibody kits using recombinant thyroid peroxidase. Positive distributions demonstrate greater than 98% concordance. However, it is difficult to judge concordance in a distribution of normal human serum as a number of laboratories that use agglutination methods report weak titre (1/100–1/400) thyroid antibodies as positive. Such low titres may be found in many normal individuals and whilst producing a measurable agglutination cannot be considered clinically significant. It is important when reporting values that a method-specific reference range, provided with kit reagents or derived from normal population studies within the laboratory, is quoted. Reference ranges are not always consistent between different methods and reagents.

Quantitative results for the agglutination assays available from Murex and Serodia show a significant difference between their dose–response relationship, despite both methods having been standardized against the same reference preparation. Quantitative ELISA assays demonstrate a smaller group standard deviation (GSD < 1·5) than the qualitative assays (GSD > 2·0), and since the former assays are calibrated against international reference preparations results are comparable between centres.

Commercial suppliers

IMF: BD, CLS, I. Agglutination: MD, W. ELISA: BS, CLS, CG, DPC, IDS, L, SD. Recombinant TPO ELISA: CLS.

SPERM ANTIBODY

The presence of sperm antibodies reacting with antigens on the spermatozoa is considered specific and characteristic of immunological infertility.[14,15] Sperm antibodies may be either IgA or IgG, however, since IgA antibodies are rarely found without co-existing IgG antibodies, it is sufficient to screen for IgG antibodies only. Assays can be divided into those that identify specific classes of immunoglobulin, e.g. immunobead and the mixed agglutination reaction, and those that are non-specific for any immunoglobulin isotype, e.g. agglutination and immobilization tests. Detection of sperm antibodies may be either direct, using fresh untreated semen, or indirect, using complement inactivated serum from either male or female origin. Some centres consider that in males, if the antibody is not present in the seminal plasma, then the serum assay is irrelevant. The principle of direct agglutination will be discussed with respect to the mixed antiglobulin reaction

(MAR) test. All other information will relate to indirect serum analysis.

Reference material
Several reference preparations of autoimmune antibody to human spermatozoa (69/65, 69/82, 69/80) are available from NIBSC.

Assay types
Most laboratories use agglutination assays involving inert particles. Several different forms of particle are used including beads, latex, etc.[16] The majority of laboratories using commercial assays use one of two kits (see Quality assurance below) which are bead-based but have an apparent lack of sensitivity to detection of IgM antibodies. The kit produced by Microm UK (Sperm MAR) is recommended by the Basic Semen Analysis courses and is reliable and easy to use.

The principle of both the direct agglutination reaction, using semen, and the indirect reaction, is that anti-sperm antibody cross-links inert particles to the sperm, causing inhibition of movement or in some cases complete immobilization of the sperm.

Direct agglutination
Fresh, untreated semen from the patient is mixed with the particles coated with human IgG. Monospecific anti-human IgG is added and the slide is observed under the light microscope after 2–3 min. The anti-human IgG binds to the IgG on the particle, in the presence of sperm antibodies the antibody will bind to the sperm and also with the anti-human IgG so that the particle becomes attached to the sperm. A negative test is shown by the sperm moving freely uncovered by the particles. The particles agglutinate, proving the reactivity of the reagents and hence acting as an internal control. The presence of anti-sperm antibody is suspected when 10–39% of the motile sperm are covered by the inert particles. If greater than 40% of the sperm are coated with particles a diagnosis of immunological infertility, due to anti-sperm antibody, is highly probable.

Indirect agglutination
This assay requires a fresh semen sample from an antibody negative donor with motile sperm present in the semen. To check for the presence of motile sperm an aliquot of semen is centrifuged to form a pellet and Earle's medium or gamete preparation medium is layered on top and the sperm allowed to swim up into the medium. The number of motile sperm is adjusted to give 2×10^7 sperm/mL. Samples with low sperm count or poor mobility may give false negative results.

A complement inactivated plasma or serum sample is incubated with the fresh motile donor sperm. If the sample contains anti-sperm antibodies, these will bind to the donor sperm and produce a positive reaction in a subsequent direct assay. Greater than 40% binding is the lower limit of significant activity in an indirect assay.

ELISA
ELISAs are now available for the detection of anti-sperm antibodies on sperm and in serum, however, initial results would suggest that these assays are less reliable than the agglutination assays.[17] All classes of antibody can be detected. As more becomes known about sperm antigens, these assays may become more clinically useful.[18]

Assay characteristics
Commercial suppliers report intra-assay coefficients of variation of 10–20% with ELISA assays.

Quality assurance
UKNEQAS for sperm antibody detection is organized from the Sub-fertility Laboratory, St Mary's Hospital, Manchester. Laboratories participating in the scheme measure sperm antibodies using either tray agglutination ($n = 10$), immunobead ($n = 5$), MAR ($n = 4$), gel agglutination ($n = 1$) or ELISA ($n = 1$).

Results are reported as positive or negative. Negative distributions demonstrate between 91–95% concordance. Positive distributions demonstrate 82% concordance.

Commercial suppliers
Agglutination: M. ELISA: L, MN, M.

ANTIBODIES ASSOCIATED WITH THE STOMACH

Antibodies to gastric parietal cells and intrinsic factor, a secretory product of the parietal cell, are strongly associated with autoimmune (type A) gastritis and pernicious anaemia.[19,20] Loss of parietal cells and a decrease in intrinsic factor secretion is characteristic of pernicious anaemia suggesting that both antibodies may be directly involved in the disease process.

Used together the two antibodies are highly specific (90%) for autoimmune gastritis and pernicious anaemia.

Gastric parietal cell antibody

Gastric parietal cell (GPC) antibodies are often (40%) found in associated organ-specific autoimmune disease, e.g. thyroid disease, and are present at low titres in 10–15% of elderly patients without any evidence of overt autoimmune disease.

Reference material

There are no national or international reference preparations available for gastric parietal cell antibody, although positive control serum samples are available from several commercial sources.

Assay type

Indirect immunofluorescence using gastric fundus from rat, human or mouse stomach remains the most widely used method for detecting gastric parietal cell antibody, although a small number of commercial companies produce ELISA kits.

Assay characteristics

The GPC antibodies react only with the parietal cells of the stomach, producing a fine meshwork appearance. These antibodies are directed against either an antigen present on the parietal cell, recently recognized as H^+ and K^+ ATPase,[21] or to intrinsic factor.

Heterophile antibodies present in the sample may produce false positive results on rat stomach parietal cells;[22] for this reason a number of laboratories prefer to use mouse stomach.

Differentiation of the GPC pattern from the similar staining pattern produced by antimitochondrial antibodies (AMA) in the stomach is done by consideration of the kidney section that forms part of the composite block, the kidney tubules will be fluorescent in the presence of AMA but not in the presence of GPC antibodies.

Quality assurance

The UKNEQAS reports only qualitative responses for gastric parietal cell antibody. Quantitative analysis is not clinically useful. Negative distributions demonstrate between 89–97% concordance; positive distributions demonstrate approximately 80% concordance. This would suggest a lack of sensitivity in some laboratories which will be directly due to the variability in the composition of the antigenic sources and the conjugate used. Alternatively, these differences could reflect false positive results in some laboratories. No laboratories participating in the UKNEQAS are using ELISA assays for the detection of gastric parietal cell antibody.

Commercial suppliers

IMF: A, BS, BD, CLS, IDS. ELISA: CG, L.

Intrinsic factor antibody

Antibodies directed against intrinsic factor may be subdivided into two different types.[23] Type I, a blocking antibody, hinders the binding of vitamin B12 to the intrinsic factor molecule inhibiting intrinsic factor–vitamin B12 complex formation. Type II, a binding antibody, binds to both intrinsic factor and the intrinsic factor–vitamin B12 complex and prevents adsorption. Both antibodies are involved in the pathogenesis of pernicious anaemia. However, since type II antibodies rarely occur in the absence of type I antibodies, quantification of both is rarely required. Intrinsic factor antibodies are a more specific marker for pernicious anaemia than gastric parietal cell antibody alone.

Reference material

There are no national or international reference preparations available for intrinsic factor antibody.

Assay types

The nature of type I antibodies led to the development of a competitive radio binding assay, in which intrinsic factor antibody present in the sample competes with radiolabelled vitamin B12 for binding to intrinsic factor. Samples are incubated with a fixed volume of gastric juice containing a known concentration of intrinsic factor. Any intrinsic factor antibodies present in the sample will form complexes with the intrinsic factor present in the gastric juice and block any subsequent binding of labelled vitamin B12. Free vitamin B12 is precipitated out with a 2% charcoal suspension and the activity of the label measured in the pellet. Results are expressed as an arbitrary unitage since no international standard exists.

Exogenous sources of vitamin B12, given either as treatment or as part of the Schilling test, must be adsorbed out of the sample using 4% charcoal as this will cause interference.[24]

ELISA detects both type I and type II antibodies and is, more importantly, unaffected by endogenous vitamin B12. Results are reported either qualitatively, positive or negative, or semi-quantitatively as a binding index.

Assay characteristics
Most of the radio binding assays are non-commercial and involve the laboratory in complex preparation and standardization of intrinsic factor from gastric juice, prior to its use in the assay, whereas ELISA has the advantage of being simpler and less time-consuming. Commercial suppliers of ELISA kits report inter-assay coefficients of variation of 7–11%.

Quality assurance
No quality assurance scheme exists at present.

Commercial suppliers
ELISA: BD, CLS (both are marketing the same kit manufactured in the USA).

ANTIBODIES ASSOCIATED WITH THE INTESTINE

Coeliac disease is a permanent intolerance to gliadin (gluten) leading to intestinal villus flattening in susceptible subjects. A number of serological markers have recently been incorporated into the revised criteria for the diagnosis of coeliac disease by the European Society of Paediatric Gastroenterology and Nutrition. Serological screening for antigliadin, reticulin and endomysial antibodies offers non-invasive tests for the investigation of coeliac disease, and varying degrees of sensitivity and specificity have been reported.[25,26] Compliance with a gluten-free diet results in a rapid decrease in IgA gliadin, endomysial and reticulin antibody concentrations, with complete avoidance for longer than 9 months leading to reduced or negative antibody concentrations.

Laboratory request patterns would suggest that there is some confusion as to which antibodies should be measured for the investigation of coeliac disease. It appears that IgA is the main mediator of the clinical manifestations of the coeliac disease process and that IgG antibodies only coexist in the presence of IgA antibodies. Patients with IgA deficiency show an increased incidence of coeliac disease and cannot be expected to develop IgA antibodies. In this group investigation for the presence of IgG antibodies is therefore justified.[27]

IgA gliadin and IgA endomysial antibodies are sensitive and specific markers for coeliac disease. Reticulin (R1) antibodies are directed against a number of antigens, one of these the so-called R1 is highly specific but relatively insensitive for disease (25–30%). Endomysial IgA antibodies provide the most sensitive (approximately 90%) and specific (96%) marker for coeliac disease and the associated skin manifestations of dermatitis herpetiformis. There is also good correlation (approaching 100%) with diagnosis achieved from biopsy material. As a financial consideration measuring either IgA gliadin and/or IgA endomysial antibodies may be the most cost-effective approach to serological screening for coeliac disease.

The presence of IgA gliadin antibody in saliva has been reported as a less invasive investigation.[28] Results show good sensitivity and specificity for salivary IgA gliadin antibody for screening for coeliac disease but there is a poor correlation between serum and salivary IgA antigliadin antibodies in patients with untreated coeliac disease.

Reference material
There are no reference preparations available for either endomysial or reticulin antibodies, but positive control serum samples are available from several commercial sources.

In the absence of an internationally available reference preparation for antigliadin antibody, the commercial suppliers have produced calibrators. These have been assigned values relative to their own preparations of affinity purified antigliadin antibodies, which have been isolated from the serum of patients with coeliac disease.

The Pharmacia and CLS kits determine the concentration of antibody by single point calibration through the origin with results expressed as EU/mL. Values greater than 25 EU/mL are scored positive. The Binding Site, however, provides a set of calibrators to prepare a standard curve, and results are expressed in mg/L of specific antibody.

Assay types
Endomysial and reticulin antibodies
Both endomysial and reticulin antibodies are directed against 'reticulin-like', silver stain positive fibres in connective tissues.

Indirect immunofluorescence is used to screen for anti-endomysial and anti-reticulin antibodies on a number of different tissues. These include

sections of rat or mouse kidney for reticulin antibody and monkey oesophagus and human umbilical cord for endomysial antibody. The use of monkey tissue requires the addition of non-immune serum, from the same species as the conjugated antibody, to reduce non-specific binding of the conjugate to the tissue. Samples are screened at a fivefold dilution.

Antigliadin antibodies
There are a number of commercially available ELISA kits for assaying both IgA and IgG antigliadin antibodies. The main difference between these assays is the units in which results are expressed (see reference materials).

Assay characteristics
Endomysial antibodies
Endomysial and smooth muscle antibody staining must be clearly distinguished. Endomysial antibodies produce a network of thin, irregular lines around the sarcolemma of the individual smooth muscle fibrils on monkey oesophagus, as opposed to smooth muscle antibodies which react with the sarcoplasm. The specific endomysial antigens occur in primate but not in rodent tissue. Umbilical tissue has only recently become recognized as a possible tissue for endomysial antibody[29] with advantages of easy availability and increased sensitivity. Endomysial antibodies are detected in the peritubular muscle layers of vessels, i.e. extracellular connective tissue and demonstrate a honeycomb-like fluorescence.

Occasionally prozone effects have been noticed when using low dilutions of samples with high antibody concentration. Where there is a high degree of clinical suspicion the sample should be re-analysed at higher dilution. Interference with the assay for IgA antibodies is possible by the coexistence of IgG endomysial antibodies (25% of coeliac disease patients) but the IgG antibodies are usually of low avidity and are present at low titre.

Reticulin antibodies
Reticulin antibodies may be subdivided into five different staining reaction patterns.[30] Of these, R1 is associated with gluten sensitive enteropathy and is characterized by peri-glomerular, peri-tubular and peri-vascular staining of mouse or rat kidney. Both IgG and IgA reticulin antibodies occur; although frequencies of IgG reticulin antibodies are the same or lower than IgA antibodies. IgA reticulin antibodies are more specific (59–100%) and sensitive (25–30%) than IgG, although clearly less so than endomysial antibodies as markers of coeliac disease and dermatitis herpetiformis.

Reticulin antigens are expressed in rodent but not in primate tissue. True human IgA reticulin R1 antibodies are very rarely found and most often occur with antigliadin and endomysial antibodies.

Antigliadin antibodies
Commercial ELISAs for both IgA and IgG gliadin antibodies demonstrate inter-assay and intra-assay coefficients of variation of 5–10%.

Quality assurance
The UKNEQAS for coeliac disease initially surveyed IgA and IgG antigliadin and anti-reticulin antibodies, and IgA anti-endomysial antibody.

As IgA endomysial antibody alone is reported, it is not possible to ascertain whether laboratories are measuring IgG antibodies as well.

The data for reticulin IgG antibody showed no consensus for positivity or negativity probably attributable to non-R1 specificities as discussed above. Only IgA reticulin antibodies are reported now and laboratories are not scored for their results as this is still a pilot scheme. There appears to be considerable variability in the type of tissues used, this is demonstrated by a wide range of reported sensitivities and specificities and is most noticeable for reticulin antibodies.

Most laboratories measure both IgA and IgG gliadin antibodies. The data suggest that there is a difference in the sensitivities of the different kits, attributable to different calibration.

Until an internationally recognized reference preparation is available concern remains as to the comparability of results reported from different methods.

Commercial suppliers
IMF: BD, BS, CLS, IDS. ELISA: BD, BS, CLS, IDS, L, P.

ANTIBODIES ASSOCIATED WITH THE LIVER

Several different IMF staining patterns are associated with the presence of antibodies in autoimmune liver disease. The most commonly recognized are mitochondrial antibody, smooth muscle antibody and liver/kidney microsomal antibody. The disease associations of the antibody staining patterns are various as shown in

Table 3. The titre of the antibody is of some clinical significance.

Antimitochondrial antibody

High titre mitochondrial antibodies are associated with primary biliary cirrhosis (>95%);[31] however weaker titre antibodies may be found in association with chronic active hepatitis,[32] mechanical bile duct obstruction and several other autoimmune disorders.[33]

A number of antigens have been identified as mitochondrial components, mainly enzymes, against which the mitochondrial antibodies are directed. These can be subdivided clinically into three groups (Table 4): those associated with primary biliary cirrhosis (PBC), those associated with hepatitis, and those found in a number of other conditions. Very little is known about autoantibody involvement in the aetiology and/or pathogenesis of associated diseases.

Anti-smooth muscle antibody

Antibodies to smooth muscle are associated, in high titre, with hepatitis B surface antigen (HBsAg) negative chronic active hepatitis[34] but may be detected at lower concentrations in patients with PBC and after infection.

Smooth muscle antibodies directed against G-actin are more specifically associated with autoimmune liver disease, whereas smooth muscle antibodies directed against other cellular filaments are more commonly associated with viral hepatitis.[35]

Anti-liver/kidney microsomal antibody (LKM)

Several sub-types exist[36 38] (Table 4) which may be distinguished by the experienced eye on indirect immunofluorescence. The most important of these is LKM-1 which reacts with a cytochrome P450 mono-oxygenase, P4502D6 and is associated with autoimmune chronic active hepatitis. A number of patients with hepatitis C have also been found to have this form of liver/kidney microsomal antibody,[39] and since the treatment for infectious and autoimmune hepatitis are different, the development of assays for determination of the subtypes of the LKM-1 antigen would have clinical application. Other subtypes are associated with cryptogenic cirrhosis and drug-induced hepatitis, but these are less well characterized.

Reference material

A reference preparation for anti-mitochondrial antibody is available for qualitative screening as primary biliary cirrhosis serum 67/183 from NIBSC. A WHO International Reference serum for anti-smooth muscle (anti-actin) antibody is available from the CLB, Amsterdam.

Assay types

Indirect immunofluorescence using rat liver, kidney and stomach allows distinction between the different antigen specificities. The assays are at best semi-quantitative with limitations as discussed previously.

The subtypes of mitochondrial antibodies are differentiated using purified antigens in antigen-specific ELISA, as minor differences in immunofluorescent staining patterns are very difficult to interpret. These assays have a limited commercial market with only ELISAs for subtypes M2, M4 and M9 available.

The LKM subtypes are also distinguished by ELISA assays. At present these are not available commercially and are predominantly research assays.

The disease-specific subgroups of anti-mitochondrial and liver/kidney microsomal antibodies are not routinely investigated and at present, until their usefulness is better established, they are best measured in specialist

TABLE 3. *Antibodies associated with autoimmune liver disease*

Antibody	Disease association	Specificity
Anti-mitochondrial	Primary biliary cirrhosis	>95%
	Chronic active hepatitis	30%
	Mechanical bile obstruction	2–3%
Anti-smooth muscle	Primary biliary cirrhosis	50%
	Chronic active hepatitis	70%
Anti-liver/kidney microsomal	Chronic active hepatitis Early cryptogenic cirrhosis Drug-induced hepatitis	Uncommon but specific

TABLE 4. *Antigen specificities and disease associations of the subtypes of anti-mitochondrial antibodies (M) and liver/kidney microsomal antibodies (LKM)*

Antibody	Antigen	Disease association
Mitochondrial antibodies		
PBC-associated antibodies		
M2	E2-pyruvate dehydrogenase complex	Highly specific for PBC
M4	Sulphite oxidase	Poor prognosis into terminal cirrhosis
M8		
M6		
M9	Glycogen phosphorylase	PBC-slow and good prognosis
Hepatitis associated antibodies		
M3		Pseudo-lupus
M6		Drug-induced hepatitis
Other disease associated antibodies		
M1	Cardiolipin	Syphilis
M5		Collagenoses
M7	Sarcosine dehydrogenase	Acute/chronic cardiomyopathy
Liver/kidney microsomal antibodies		
LKM1	Cytochrome P450 2D6	Autoimmune CAH,[36] hepatitis C
LKM2	Cytochrome P450 2C8/9/10	Drug-induced hepatitis[37]
LKM3	Uridine diphosphate glucuronyl transferase	Chronic δ virus hepatitis[38]

PBC = primary bilary cirrhosis.

centres involved with the investigations of liver disease.

Assay characteristics

Anti-mitochondrial antibodies

Mitochondrial antibody appears as discrete granular staining, predominantly of the distal tubules of the kidney and parietal cells of the stomach.

Anti-smooth muscle antibodies

Smooth muscle antibodies react with the muscle fibres in the walls of the arteries, in the intergastric gland area of the *lamina propria* in the stomach and in the *muscularis mucosa* layers of the stomach. At high titres they may react with tubulin in the glomerular basement membrane.

Anti-LKM antibodies

Antibodies to liver/kidney microsomes react with a 'feathery' appearance in the hepatocytes of the liver and the proximal tubules of the kidney, but not in the stomach.

A composite block of all three tissues prevents misclassification of the liver/kidney microsomal antibody and anti-mitochondrial antibody. The main difference is the binding of antibody to the kidney tubules: liver/kidney microsomal antibody reacts predominantly with the proximal tubules whereas anti-mitochondrial antibody reacts predominantly with the distal tubules. However, in practice these reactions are not mutually exclusive and classification will depend on the intensity of the staining pattern being stronger in one or other of the tubule types. It is obviously important when preparing tissue sections that an appropriate cross-section of the kidney is cut so that both types of tubule are present in each tissue slice.

Quality assurance

The UKNEQAS reports qualitative responses for smooth muscle antibody and anti-mitochondrial antibody, with a quantitative titre reported for positive anti-mitochondrial antibody. Negative distributions to anti-mitochondrial antibody have shown greater than 95% concordance, positive distributions have shown greater than 91% concordance, with varying degrees of semi-quantitative titre. Most laboratories use either mouse or rat tissue, but no information is given on the source or preparation of these tissues. Problems have arisen when unusual staining patterns have been distributed, one such distribution included an atypical liver reactive antibody which 26% of laboratories falsely reported as anti-mitochondrial antibody: this was not a M2 mitochondrial antibody nor a liver/kidney microsomal (LKM-1) antibody. There is considerable difference in pattern interpretation and we would advise that where unusual staining patterns occur laboratories

refer samples to more experienced centres for their interpretation. Quantification of the subtypes of anti-mitochondrial antibody are not reported by the UKNEQAS, so no data are available on their performance.

Negative distributions of anti-smooth muscle antibody demonstrate greater than 88% concordance, with over-sensitivity the main concern. This may be due to variation in screening dilutions between laboratories. In our experience a screening dilution of 1/20 will eliminate any results which are not clinically significant.

No UKNEQAS exists for liver/kidney microsomal antibody but where this pattern occurs it should be reported.

Commercial suppliers

IMF: BD, BS, CLS, IDS, I. ELISA: Anti-mitochondrial antibody: M2—BD, CG, IDS, L; M4—BD; M9—BD. Liver/kidney microsomal antibody: LKM1—L.

EPIDERMAL/SKIN ANTIBODIES

A complete investigation of the bullous skin diseases should include both direct immunofluorescence of biopsy material and indirect serum immunofluorescence, since each investigation alone may give false negative results.[40]

For the purposes of this review we will only consider the serum antibodies detected in these conditions. These are of two types, intra-epidermal/desmosome antibody (pemphigus antibody) associated with all forms of pemphigus, and basement membrane zone antibody (pemphigoid antibody) associated predominantly with bullous pemphigoid but occasionally detected in variants of this disease. These antibodies are present in the serum of 70–90% of affected individuals. The pemphigus antibody concentration correlates with disease activity and disappears during clinical remission, whereas the pemphigoid antibody is not directly pathogenic, titres do not correlate with disease activity and antibodies may still be found in serum and tissues in clinical remission.[41]

Reference material

There are no reference preparations available for either pemphigus or pemphigoid antibodies; however, positive control serum samples are available from several commercial suppliers.

Assay types

Both antibody specificities can be detected by indirect immunofluorescence of a single tissue; however, there are a number of different tissues available. These include monkey, human, guinea pig, rat and mouse oesophagus, human skin and guinea pig lip and tongue. Most of these tissues are available from commercial sources and are sometimes used in combination to achieve the best clinical sensitivity and specificity. Most laboratories restrict testing to the demonstration of the IgG class for both antibodies although IgA basement membrane antibodies may be clinically significant in other bullous skin disorders.

Assay characteristics

Pemphigus antibodies react with antigens present on the cell surface of epidermal keratinocytes, giving a characteristic 'chicken-wire' staining pattern around but not within the keratinocytes. The pemphigus antibodies have been shown to react directly with a 130 kD glycoprotein, desmoglein 3.[42]

Pemphigoid antibodies produce a linear deposition of antibody along the dermo-epidermal junction. The antibody associated with bullous pemphigoid and herpes gestationis has been shown to react with a 180 kD transmembrane protein, BP180, but more specifically with MCW-1, an immunodominant epitope on this protein.[43]

ELISAs have been established for these antigens but are only available for research purposes at the present time.

Quality assurance

The UKNEQAS for skin antibodies reports qualitative results for desmosome and basement membrane zone antibodies. Results suggest that greater than 80% of laboratories can detect these antibodies reliably.

Commercial suppliers

IMF: BD, BS, CLS, IDS

ANTIBODIES ASSOCIATED WITH NEUROMUSCULAR DISORDERS

Acetylcholine receptor antibody

Acetylcholine receptor antibodies (AChR) are associated with myasthenia gravis. There are three types of AChR antibodies: binding antibodies, blocking antibodies and modulating antibodies. Although antibody concentrations do not correlate well with disease activity, they may be used to monitor plasma exchange therapy.[44]

Reference material
There are no reference preparations available for acetylcholine receptor antibody.

Assay types
A competitive binding assay that uses detergent-solubilized human acetylcholine receptor labelled with [^{125}I]α-bungarotoxin is the standard test for anti-acetylcholine receptor antibody.[45] Radiolabelled bungarotoxin along with any anti-AChR antibodies present in the sample bind specifically to the acetylcholine receptor. These complexes can then be separated from non-antibody bound AChR by solubility in 2% polyethylene glycol, or by a second antibody to human IgG. The degree of precipitated radioactivity is a measure of the antibody activity. Results are expressed in nmol/L of bungarotoxin binding activity, or as a percentage of a standard positive serum.

ELISAs have been developed but the antibodies do not bind well to the immobilized acetylcholine receptor.

Assay characteristics
These assays are best restricted to specialized centres experienced in their investigation. Preparations of the acetylcholine receptor from human skeletal muscle can vary, causing batch-to-batch variation in the number of receptors present and hence the bungarotoxin binding. More consistent results may be obtained using cultured cell lines (TE671) as a source of the receptor protein.[46]

Quality assurance
Acetylcholine receptor antibody samples have a small distribution through the UKNEQAS, as part of the European NEQAS. There are only five centres registered in the UK. Laboratories use either commercial kits or have their own laboratory method. Results are reported both qualitatively and quantitatively. Laboratories will soon be scored using the misclassification index (MIS). Distributions to date would suggest that there are problems with calibration due to the lack of any reference material.[47]

Commercial suppliers
RIA: IDS, RSR.

OTHER ANTIBODIES FOUND IN MYASTHENIA GRAVIS

Skeletal/striated muscle antibodies often coexist with acetylcholine receptor antibodies, although the acetylcholine receptor antibody is most well known and the most often measured[48]. Striated muscle antibody shows very characteristic striations on muscle tissue when viewed by indirect immunofluorescence. Laboratories preparing their own rat or mouse tissue sections have the choice of a wide range of muscle tissue including abdominal muscle for this purpose; heart muscle is probably the most commonly used. Primate tissue is preferable as it is the most antigenically similar to human tissue and will result in less false negatives.

Other tests that measure accelerated degradation or blockade of acetylcholine receptors are sometimes helpful in antibody negative myasthenia gravis.[49]

ANTIBODIES ASSOCIATED WITH OTHER NEUROLOGICAL DISORDERS

Other neurological autoantibodies of interest include anti-ganglioside antibody (anti-GM1), the anti-neuronal antibodies including Purkinje-cell antibody, anti-Yo antibody and anti-Hu antibody and the voltage-gated calcium channel antibodies.

Anti-GM1 antibody is associated with a motor neuropathy causing multifocal conduction blocks. Both IgM and IgG antibodies are clinically useful although of limited disease specificity.[50]

Voltage-gated-calcium channel (VGCC) antibodies are associated with the Lambert Eaton myasthenic syndrome. Assays for these antibodies are still under development and their clinical usefulness has yet to be evaluated.[51]

The assays for the above antibodies are restricted to specialized centres with a particular interest in these disorders.

KIDNEY ASSOCIATED ANTIBODIES

Glomerular basement membrane antibody (GBM)
Glomerular basement membrane antibodies are directed against the non-collagenous portion (NC1) of type IV collagen and are detected in classical, untreated Goodpasture's syndrome.[52] The classic presentation of anti-GBM disease is rapidly progressive glomerulonephritis (RPGN). The antigen shares common epitopes with renal and pulmonary alveolar basement membranes which may suggest why patients can present with rapidly progressive immune complex disease causing pulmonary haemorrhage and haemoptysis. The presence of GBM antibodies, using the specific antigen ELISAs, is diagnostic for

Goodpasture's syndrome and permits differentiation of Goodpasture's syndrome from other causes of pulmonary haemorrhage and glomerulonephritis. Early diagnosis is important as untreated patients will suffer extensive tissue damage which in extreme cases will result in death. High titre anti-GBM responses may persist even in clinical remission and decrease slowly over several months. Renal transplantation should only be considered in antibody negative patients to avoid recurrence of the disease.

The coexistence of glomerular basement membrane antibody and anti-neutrophil cytoplasmic antibody in patients with systemic vasculitis is becoming increasingly recognized and will be discussed further in the section on anti-neutrophil cytoplasmic antibody.

Reference material

There are no reference preparations available for glomerular basement membrane antibody. Positive control serum samples are available from several commercial suppliers.

Assay types

Tissues used in indirect immunofluorescence include rat, mouse or primate kidney: the latter is more closely related in antigen expression to the human resulting in a more specific assay.

Both qualitative and quantitative ELISA kits are available. Biodiagnostics offer a multi-antibody nephrology screening kit for GBM and anti-neutrophil cytoplasmic antibody specificities (see ANCA section). The qualitative kits are used predominantly for screening, with all positive results verified by quantitative assays.

Assay characteristics

The indirect immunofluorescence technique is less sensitive and specific than the ELISA assays, and gives positive results in only 75% or fewer cases of confirmed Goodpasture's syndrome.[53]

False positive results are common using human and monkey kidney and occur in conditions such as SLE where there is polyclonal increased IgG. True linear staining due to anti-GBM antibody is difficult to distinguish from non-specific staining, so ELISA assays are the method of choice as the specific antigen can be used.

The sensitivity and specificity of ELISA depend on the purity of the antigen preparation. Most assays now use the C-terminal end of type IV collagen where previously crude extracts of glomerular basement membrane were used.

Quality assurance

No quality assurance scheme exists for the detection of anti-glomerular basement membrane antibody at present.

Commercial suppliers

IMF: BD, BS, CLS. ELISA: BD, SD.

ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY

Anti-neutrophil cytoplasmic antibodies (ANCA) are a family of autoantibodies, directed against lysosomal enzymes of myeloid cells, and are associated with vasculitic disorders and glomerulonephritis.[54] These diseases have a wide range of clinical presentations which, if undiagnosed, can lead to progressive and irreversible tissue damage to the kidney and lungs. It has been reported that the concentration of antibody correlates with disease activity,[55] and that in many patients the concentrations return to normal in remission, only becoming positive again if the disease relapses.

Quantification, however, remains an area of controversy. The two most commonly identified staining patterns are c-ANCA (cytoplasmic or classical staining) and p-ANCA (perinuclear staining). These are identified by indirect immunofluorescence, the recommended screening method for the detection of ANCA.[56] In ANCA associated systemic vasculitis the predominant antibody specificities are proteinase III for c-ANCA[57] and myeloperoxidase for p-ANCA.[58] There are a number of cases where the clinical significance of the antibody suggests that the methods of choice for detection of these antibodies should be antigen specific ELISA and radioimmunoassay assays. More recently flow cytometry has been developed for the quantification of ANCA specific antigens, but this is restricted to a few centres only and is therefore difficult to evaluate as a routine method.[59]

Testing for both glomerular basement membrane antibody and ANCA is indicated in the investigation of RPGN and crescentic glomerulonephritis. In these patients the presence of GBM antibody has a poorer prognosis than patients with the c-ANCA associated antigen proteinase III.[60] In patients presenting with reno-pulmonary syndromes a positive GBM autoantibody or positive ANCA will exclude infection or malignancy.

Reference materials

A number of commercial serum samples for use in internal quality control procedures are

available for both c-ANCA and p-ANCA and also from PRU Procurement.

A qualitative international reference serum for anti-proteinase III exists but has not yet been used in an attempt to standardize quantitative assays.

Commercially available ELISA kits for quantification of either myeloperoxidase or proteinase III use arbitrarily assigned values chosen by the manufacturer for calibration. Reference limits are assigned and their limitations quoted.

Assay types

Indirect immunofluorescence

Purified human neutrophils fixed in ethanol are used as the primary screen by indirect immunofluorescence.[61] These can be prepared in the laboratory or obtained from a number of commercial sources.

Preparation of cells involves separation of neutrophils from fresh anticoagulated (heparin) blood by density gradient sedimentation followed by cytocentrifugation of the neutrophil suspension into wells on glass slides. The slides are then fixed in ethanol or formalin, before drying and storage with desiccant at −40°C. Although the preparation of the slides is quite labour intensive, the reagent costs are less than the purchase price of commercial slides.

ELISA

Since the major antigens associated with ANCA have been identified, a number of solid phase assays for the detection and quantification of ANCA have been established. However, ELISA which use crude neutrophil extracts as antigenic source are no more sensitive or specific than IMF. ELISA using α-granule extracts may contain antigens other than proteinase III.

Assay characteristics

Immunofluorescence

On ethanol-fixed neutrophils two types of immunofluorescent staining patterns can commonly be demonstrated, the cytoplasmic or c-ANCA pattern and the perinuclear or p-ANCA pattern. The c-ANCA stains in a granular pattern with a central interlobular accentuation, whereas the p-ANCA shows an intense staining in the perinuclear space. The p-ANCA pattern is, however, an artefactual staining of alcohol-fixed neutrophils caused by positively charged granule proteins being attracted to the negatively charged DNA in the nucleus. This pattern may also be caused by the presence in the sample of anti-nuclear antibodies (ANA), especially those directed against the dsDNA or histone antibodies. Granulocyte specific ANA (GS-ANA) positive samples also produce a nuclear or perinuclear reaction on ethanol fixed neutrophils.

For this reason all samples producing these types of staining pattern should be retested using formalin-fixed neutrophils. Using formalin as a fixative, the antigens responsible for the p-ANCA pattern are preserved in their original location in the azurophil granules, giving a granular cytoplasmic staining pattern. Antinuclear antibodies are abolished or reduced in intensity by formalin fixation. For a summary of staining reactions see Table 5.

In addition to myeloperoxidase, other antigens may also be associated with the p-ANCA staining pattern: these include elastase, lactoferrin, and cathepsin G, and demonstrate the lack of disease specificity for this staining pattern.

Other staining patterns have been reported including atypical p-ANCA, atypical c-ANCA and x-ANCA. The significance of these is unknown.

All titrations should be performed on ethanol fixed slides, since titration on formalin-fixed neutrophils is unreliable and is not recommended. Where coexisting ANA and p-ANCA patterns are found, the p-ANCA titre is only valid if it exceeds the ANA titre by at least two dilution steps.

TABLE 5. *Staining patterns of the various antibody types on the three types of cells, ethanol-fixed neutrophils, formalin-fixed neutrophils and Hep-2 cells*

Antibody type	Ethanol-fixed	Formalin-fixed	Hep-2 cell
c-ANCA	Cytoplasmic	Cytoplasmic	None
p-ANCA	Nuclear/perinuclear	Cytoplasmic	None
ANA	Nuclear/perinuclear	None	Nuclear
GS-ANA	Nuclear/perinuclear	None	None
p-ANCA & ANA	Nuclear/perinuclear	Cytoplasmic	Nuclear

ANCA = anti-neutrophil cytoplasmic antibody; c-ANCA = cytoplasmic staining; p-ANCA = perinuclear staining; ANA = antinuclear antibody; GS-ANA = granulocyte specific.

ELISA

ELISA is becoming the method of choice for ANCA detection as it can be more readily quantifiable and has increased antigen specificity, therefore offering a better tool for monitoring disease activity. Commercial ELISA report inter-assay coefficients of variation of 5–11% and intra-assay coefficients of variation of 4–11%.

Quality assurance

UKNEQAS organizes a qualitative and quantitative scheme for the detection of ANCA. The largest single group are those laboratories (~37%) preparing their own slides; 32% of laboratories screen by indirect immunofluorescence and follow-up positive staining patterns with the relevant specific antigen ELISA, whilst only 8% use ELISA alone. Those laboratories using ELISA demonstrate >95% concordance. Results for qualitative screening for ANCA demonstrate good sensitivity and specificity with most problems occurring with the identification of the p-ANCA pattern as opposed to the c-ANCA pattern.

Commercial suppliers

IMF: BD, BS, CLS, IDS. ELISA: BD, BS, CLS, IDS, N, SD.

RHEUMATOID FACTOR

Rheumatoid factor is an autoantibody directed against the Fc portion of immunoglobulin G (IgG)[62] and is a significant serological marker of rheumatoid arthritis. The most commonly measured and clinically useful class of rheumatoid factor is IgM. ELISA assays now exist for the detection of IgA and IgG but these have yet to find their place in the diagnosis and monitoring of rheumatoid arthritis. In rheumatoid arthritis IgA rheumatoid factor is reported to be associated with bone erosions.[63] A positive or negative rheumatoid factor respectively neither confirms nor excludes rheumatoid arthritis, since IgM rheumatoid factor is found in 2–10% of apparently healthy adults.

Reference material

An international reference preparation for rheumatoid factor is available from the NIBSC (64/2). In order to facilitate the inter-laboratory interpretation of results, it is advisable that all results are reported in IU/mL based on the WHO reference standard (64/1) available from the CLB, Amsterdam.

Assay types

Rheumatoid factor reacts more strongly with aggregated IgG than with native human IgG; therefore all assays use aggregated human gamma globulin as the antigen.

The methods available for the measurement of rheumatoid factor can be divided into three distinct groups: Instrument-dedicated methods using either nephelometry or turbidimetry, enzyme immunoassay which measure rheumatoid factor quantitatively and are calibrated against a WHO reference preparation, and agglutination assays which measure rheumatoid factor semi-quantitatively with positive results reported as titre.

Agglutination

Historically the Rose–Waaler test was widely used.[64,65] Most laboratories now use a simple latex agglutination test[4] to screen for positive samples. Presence or quantification is then by a more specific test. The Rose–Waaler test uses rabbit IgG which is incubated with sheep red blood cells to produce sensitized cells, which will agglutinate when IgM rheumatoid factors react with the rabbit IgG. Non-specific anti-sheep red cell antibodies are detected by preparing unsensitized cells as a control. However, this is a complex and time consuming assay and test samples must be heated at 56°C for 30 min to inactivate complement. The latex agglutination method uses latex coated with human IgG and these particles will be agglutinated by IgM rheumatoid factor. Immunoglobulin sensitized gelatin particles may also be used with the same principle.

ELISA

In the enzyme immunoassay bound antibody is detected with an enzyme conjugated anti-human immunoglobulin. All the commercial ELISAs listed in this article measure IgM rheumatoid factors; however, there are ELISAs available for the measurement of IgA and IgG rheumatoid factor. ELISAs have the advantage of providing a readout on a continuous scale.

Assay characteristics

Latex agglutination assays produce reliable results if used in controlled conditions and serve as quick and cheap screening tests. However, they give a number of false positive results and so should be used in conjunction with the more specific red cell agglutination assay. Positive samples are therefore tested in the Rose–Waaler assay using red cells prepared in the laboratory or by using a commercially prepared kit.

A MDD report on the evaluation of eight commercial kits for rheumatoid factor detection and quantification reviews the existing automated and ELISA assays for IgM rheumatoid factor. It advises that most kits demonstrate reasonable linearity, but the Boehringer system demonstrated a positive prozone effect. All assays demonstrated within assay and between assay variability, at three concentrations, of less than 3% and less than 8% respectively. Occasionally, the presence of circulating immune complexes may cause discrepancies between different methods.

Quality assurance

The UKNEQAS reports rheumatoid factor both qualitatively and quantitatively. Both negative and strong positive distributions demonstrate greater than 95% concordance. The liquid phase assays, which are calibrated against WHO reference preparations, demonstrate a much smaller GSD ($<1{\cdot}6$) than the agglutination assays (GSD $>2{\cdot}2$) on both low titre positive and positive sera. Whilst those agglutination assays calibrated against an international reference preparation (IRP) demonstrate an intermediate response (GSD $\sim 2{\cdot}0$).

The agglutination assays demonstrate considerable inter-manufacturer variability in their dose–response relationship, e.g. the Serodia RAPA method demonstrates much higher titrations than other agglutination assays. This is reflected by the method-related reference range and means that assays expressing results in titres are not immediately comparable with the defined unitage of the reference preparation. Agglutination assays have also been shown to be less sensitive than the liquid phase assays.

Commercial suppliers

Particle agglutination: MD, SCAT. Nephelometry: BK, B, U. Turbidimetry: BM. ELISA: BD, CLS, CG, I, E, S, SD.

ANTINUCLEAR ANTIBODIES

Antinuclear antibodies (ANA) are a diverse group of autoantibodies found mainly in patients with systemic rheumatic diseases. The most common and clinically useful antibodies are listed in Table 6. They can be divided into two broad groups, those directed against structural or insoluble proteins, and those directed against saline and soluble antigens—the so-called extractable nuclear antigens (ENA) or soluble cellular antigens.

Screening tests for ANA most commonly employ the indirect immunofluorescence test using either frozen sections of animal tissue,[66] such as mouse kidney, or cultured cell lines such as HEp-2.[67] In addition some antigens, such as proliferating cell nuclear antigen (PCNA) and centromeric proteins are not widely expressed in rodent tissues. The antigen SSA/Ro is not conserved in its native form in preserved tissues or cell lines which may result in false negative findings in antibody positive samples. Specific testing using purified or partially purified antigen is then necessary to detect antibodies to this antigen. Both the ANA pattern and the quantified titre should be reported.

Traditionally the quantification of ANA has involved immunofluorescence using cultured cells or rodent tissue sections and serial dilutions of the sample. The titre is the highest dilution to show positive fluorescence. It has been reported that the titre correlates with disease activity in patients with SLE. To facilitate the comparability

TABLE 6. *Anti-nuclear antigen patterns and their disease associated antigens*

Pattern	Antigen	Associated diseases
Peripheral	dsDNA	SLE
Homogeneous	DNA-histone complex	SLE, occasionally other connective tissue disease
Speckled	SM	SLE
	RNP	MCTD, SLE, Sjogren's, scleroderma, polymyositis
	SSA/Ro	Sjogren's syndrome, SLE
	SSB/La	Sjogren's syndrome, SLE
	Centromere	Limited scleroderma
Nucleolar	PM-Scl	Polymyositis
	Nucleolar RNA	Scleroderma
	Scl-70	Scleroderma
Cytoplasmic	Jo-1	Polydermatomyositis

SLE = systemic lupus erythematosus; RNA = ribonucleic acid; MCTD = multiple connective tissue.

of results, the WHO has produced an international reference preparation for the standardization of titres.[68] The use of these standards has been shown to reduce inter-laboratory variability.[69]

More recently, some ELISA assays have become available to measure total ANA activity. These assays either employ cell homogenates or mixtures of purified antigens to cover the desired antibody specificities. The results of these new assays were initially poor, but recently assays giving more acceptable results have become available, although it is difficult to envisage these assays replacing the immunofluorescence screen in routine clinical practice.

The ANA test should be regarded as a screening test to identify those samples requiring specific antigen identification. The pattern of the immunofluorescence reaction will give some indication of the antigen specificity with which to rationalise further testing.[70]

Antigen preparations

HEp-2 cells (American type culture collection CCL-23) can be obtained from most suppliers of cell lines. It should be noted that there are a number of related cell lines available, and some of these may not be suitable for ANA evaluation.

Both HEp-2 and sections of animal tissues are available as pre-prepared slides from many commercial sources. These show variable sensitivity for some ANA specificities, and any potential new source should be fully evaluated before clinical use.

Reference materials

A standard serum (WHO/66/233) containing 100 IU of ANA giving a homogeneous pattern in IMF can be obtained from NIBSC and CLB, Amsterdam.

Qualitative controls for standardisation of ANA patterns are available from CDC. The following serum samples are available: AF/CDC 1, homogeneous pattern; AF/CDC 2, fine speckled pattern; AF/CDC 3, speckled pattern; AF/CDC 6, nucleolar pattern.

Assay types

Indirect immunofluorescence

IMF is the most commonly used test to screen for the presence of ANA. Increasingly laboratories are using HEp-2 cells as the target tissue. These cells have the advantage that they are large cells with large nuclei and the slide preparations will contain a significant number of dividing cells, making for easier recognition of staining patterns. Rodent tissues will reveal the presence of ANA but such tissues are poor in expression of SSA/Ro antigen and few cells are dividing so that some antibodies, which yield cell cycle-dependent patterns, will not be recognized. Monospecific antihuman IgG fluorescein conjugate is preferred since detection of IgA and IgM antibodies are rarely clinically useful and only decrease the specificity of the assay.[71]

ELISA

More recently, ELISA assays using cell homogenates, antigen mixtures or a combination of both have been developed for ANA screening.

Assay characteristics

The reproducibility and clinical relevance of results from IMF is dependent on many factors, including the cell line used, the fixation procedure, the immunochemical reagents, and the microscope used to evaluate the reaction. All of these factors should be optimized in order to obtain consistent, meaningful results. The imprecision of titres has been discussed previously. Results obtained can be converted to international units by titration of test and reference material (WHO/66/233), using the formula $P/S \times 100 = IU/mL$, where P is the titre of patient serum, and S is the titre of the standard serum. In general IMF is sensitive to about 2 IU/mL.

It has been reported that some of the ELISA assays lack the appropriate sensitivity for clinical use, but there are few published data showing direct comparisons with IMF. Commercial suppliers have reported coefficients of variations of 10–20%.

Quality assurance

Antinuclear antibody identification is included in two schemes organized by UKNEQAS. In autoimmune serology 1 both qualitative and quantitative results for antinuclear antibody and dsDNA antibody are reported, but no pattern identification is required. Distributions tend to demonstrate homogeneous staining and titres reported demonstrate GSD > 2·0. In autoimmune serology II antibodies to the nuclear related antigens are reported qualitatively together with the staining pattern seen on HEp-2 cells.

Consensus with the reference laboratories varies considerably depending on the antigen present, highlighting the true subjectivity of immunofluorescent pattern identification.

Commercial suppliers
IMF: A, BS, BD, CLS, IDS, I, Q, SD, S. ELISA: I, SD, L, N.

ANTIBODY TO DOUBLE STRANDED DNA (dsDNA)

Antibodies to dsDNA are strongly associated with SLE. Many methods exist for the quantification of anti-dsDNA. The three most commonly used are the radioimmunoassay (Farr assay), the immunofluorescent test using *Crithidia luciliae*, and more recently ELISA.[72] The majority of work on the relationship between disease activity and concentrations of anti-dsDNA has been carried out using the Farr assay. Elevated concentrations of anti-dsDNA have been reported in approximately 60% of patients with systemic lupus erythematosus (SLE).[73] The prognostic value of the measurement has been demonstrated by measuring anti-dsDNA in serial samples from patients with SLE.[74] More recently, there has been a fast growth in the number of ELISA assays available for the detection of antibodies to dsDNA. Much of the reported work has concentrated on the specificity of the assays, rather than the relationship between the concentration of antibody and disease activity.[75] One problem with the use of ELISA assays for anti-dsDNA quantification is in the detection of low affinity antibodies which are thought to be irrelevant to the disease process, and thus it is important to use assays which measure only high affinity antibodies.

Reference material
An international serum containing 200 IU/mL of anti-dsDNA is available from CLB, Amsterdam.

Assay types
Farr assay
The Farr assay is based on the precipitation by ammonium sulphate of complexes of radiolabelled dsDNA and anti-dsDNA, but not of uncomplexed radiolabelled DNA. Following incubation of radiolabelled antigen and sample, complexed material is precipitated by the addition of ammonium sulphate. The tubes are centrifuged, and the supernatant containing unprecipitated DNA is discarded. The radioactive content of the precipitate is counted, and the values obtained with the test samples are compared to those of a standard curve obtained by using a series of standards calibrated against the international standard.

Indirect immunofluorescence
IMF is commonly used to screen for the presence of antibodies to dsDNA. Cultures of the flagellate, *Crithidia luciliae* are grown in a container containing glass slides and the organism affixes to the slides. Care must be taken that the fluorescence observed is contained in the kinetoplast, and is not located within the nucleus or the basal granule, both of which may exhibit fluorescence in the absence of anti-dsDNA.

ELISA
ELISA assays using purified dsDNA as antigen have been developed for the determination of anti-dsDNA. It should be noted that the physical process of binding DNA to the solid phase may cause a distortion of the helical structure, exposing hidden epitopes and distorting conformational epitopes. These physical characteristics may account for the gross discrepancies seen with some ELISA assays.

Assay characteristics
IMF shows good sensitivity and specificity however, results are semi-quantitative making the technique applicable only to screening for dsDNA.

The ELISA methods show extreme sensitivity but reduced specificity compared with IMF. They detect antibodies of both low and high avidity and allow fully quantified results expressed in IU/mL with respect to international reference material. Coefficients of variation of 4–7% are reported by commercial suppliers of kits.

The Farr assay has the highest specificity of all the tests for SLE and the best correlation with disease activity as it only detects high avidity antibodies. Occasionally, samples demonstrate a positive Farr assay in the absence of antibodies to dsDNA. This is thought to be due to the presence of circulating non-immunoglobulin DNA binding proteins.

Of the many assay systems which have been developed for the detection of antibodies directed against deoxyribonucleic acid, each appears to detect a different spectrum of antigenic specificities.[75] Comparisons of the three techniques show a reasonable degree of concordance. Generally speaking, the ELISA assay is the most sensitive technique, whereas the *Crithidia* assay is the most specific test for the antibodies to dsDNA found in patients with SLE. Antibodies to single stranded DNA (ssDNA) are not clinically useful.

Quality assurance (QA)
The UKNEQAS report antibodies to dsDNA both qualitatively and quantitatively. It is difficult to compare quantitative results due to the number of different assays used. However, negative distributions demonstrate 93% concordance, with 82% concordance reported on positive distributions and 57% concordance on borderline positive distributions.

Commercial suppliers
RIA/Farr: JJCD. IMF: BD, BS, IDS, I, Q, SD. ELISA: BD, BS, CLS, CG, DPC, IDS, I, N, S, SD.

EXTRACTABLE NUCLEAR ANTIGEN ANTIBODIES

Antibodies to extractable nuclear antigens are a group of autoantibodies reacting with antigens which leach from the cell when extracted with saline (Table 6). A number of different methods have been developed for the detection of antibodies to ENA. These include counter-immunoelectrophoresis,[76] Ouchterlony double immunodiffusion, Western Blotting, and ELISA. The precise methodology and the relative merits of these techniques have been recently reviewed.[77,78]

Although quantification for antibodies directed against soluble cellular or extractable nuclear antigens has been performed for many years, there are few studies looking at the relationship of antibody concentration and disease activity or progression. It has been reported that the concentrations of anti-La increased during disease flares in Sjogren's syndrome.[79] However, other antibodies (e.g. anti-Sm) do not fluctuate over time. Thus, although quantification of these antibodies is possible, their clinical usefulness remains to be proven.

Reference materials
Qualitative international reference standards for the following specificities are available from CDC. AF/CDC 2, Anti-La; AF/CDC 5, Anti-Sm; AF/CDC 7, Anti-Ro; AF/CDC 9, Anti-Scl 70; AF/CDC 10, Anti-Jo-1.

In addition an international reference serum for anti-nRNP (WHO-aNRNP) is available from CLB, Amsterdam.

Assay types
Counter-immunoelectrophoresis (CIE)
CIE exploits the principle of antibodies and antigens carrying opposite electrical charges; these travel through an agarose gel in opposite directions when an electrical current is applied. Whole cell extract containing the extractable nuclear antigens is placed in a well cut into an agarose gel on a glass plate and sample is placed in a well cut in the gel opposite the antigen source. An electrical current is applied across the gel, and antigen and antibody move through the gel towards each other. If the sample contains an antibody to any of the extractable nuclear antigens, this then complexes with the antigen and a white, opaque precipitate is observed in the gel. The specificity of a detected antibody can be determined using a modified CIE, and well defined, monospecific reference sera. The preparation of gels, choice of antigen sources, and methodology has been reviewed.[76]

Ouchterlony double immunodiffusion
Ouchterlony diffusion uses an agarose gel to enable visualization of antibody–antigen complexes. Whole cell extract containing the extractable nuclear antigens is placed in a well cut into an agarose gel on a glass plate, and sample is placed in a well cut in the gel located opposite the antigen source. The plates are incubated for 48–72 h during which time antigen and antibody diffuse through the gel towards each other. If the sample contains an antibody to an extractable nuclear antigen, then this complexes with the antigen and a white, opaque precipitate is observed in the gel. The specificity of a detected antibody can be determined by the inclusion of well defined, monospecific reference sera in the assay system and the observation of 'lines of identity'.

Western blotting
Western blotting or 'immunoblotting' consists of five stages. Proteins from a whole cell extract are separated according to molecular weight on SDS–PAGE electrophoresis. The separated proteins are then transferred to a nitro-cellulose sheet. The remaining binding sites are blocked with an immunologically inert protein, e.g. human serum albumin (HSA), to prevent non-specific binding. The nitro-cellulose is then incubated with sample, and finally bound antibody is detected by incubation with an antibody to human immunoglobulin conjugated with a chromogenic enzyme, followed by a chromogenic substrate.

ELISA
ELISA assays use either recombinant or native extractable nuclear antigen peptides or immunopurified proteins as antigens. ELISA using

pooled antigens are commonly used as a screening test, before typing with the monospecific antigens. This single specificity system may avoid any problems that may occur with gel precipitates, but each system needs to be thoroughly evaluated for specificity and sensitivity before use.

Assay characteristics
ELISA demonstrate high sensitivity but with a corresponding lack of disease specificity. Commercial ELISA report inter-assay coefficients of variation of 8–14% and intra-assay coefficients of variation of 8–16%.

Counter-immunoelectrophoresis and Western blotting are both sensitive and specific for all antigen types.

Quality assurance
The UKNEQAS reports ENA specificities qualitatively. The majority of laboratories use quantitative ELISA methods (65%), with only 12% using the ENA screening kits. Negative distributions demonstrate 90% concordance and positive distributions demonstrate 73% concordance. Discrepancies occur when serum samples contain multiple antibody specificities.

Commercial suppliers
CIE: BD, BS. IMD: BD, BS. IMBT: BD, EP. ELISA: BD, BS, CLS, CG, DPC, E, IDS, I, N, Q, S, SD.

ANTI-CENTROMERE

Antibodies to the centromeric proteins are directed against epitopes found in the kinetochore domain of the chromosome. Studies report that they are associated with the CREST syndrome, a benign form of scleroderma.[80] They are most commonly detected by IMF. The choice of tissue/cell is very important as many rodent tissues do not readily express the centromeric proteins. Cultured cell lines, such as HEp-2 are usually used for their detection. More recently, an ELISA using recombinant centromeric-B peptide (CENP-B) has been developed and these assays seem more sensitive than IMF.[81]

Antibodies to the kinetochore of the centromere can be quantified either by titration by immunofluorescence or by ELISA using the reactive peptide. Initial results suggest that there is little fluctuation in concentrations of antibody over a period of time, and that quantification has little clinical application.

Reference material
A qualitative standard (AF/CDC 8) is available from the CDC.

Assay types
Indirect immunofluorescence
The assay method is identical to anti-nuclear antibody, the centromeric protein is a particular type of staining pattern seen on HEp-2 cells. Centromere antibodies give a discrete speckled pattern on cells in interphase (non-dividing) and also at the metaphase plate.

ELISA
ELISA assays using recombinant centromeric-B peptide as antigen have been developed for detection of antibodies to the centromeric antigen.

Assay characteristics
The three main centromere antigens are CENP-A, CENP-B and CENP-C, of which CENP-B is recognized by almost all samples with centromere antibodies. ELISA for CENP-B demonstrates a higher disease specificity and sensitivity than IMF. False positives in ELISA may be due to bacterial contamination. Commercial ELISA report inter-assay coefficients of variation of 4–8% and intra-assay coefficients of variation of 6–15%.

Quality assurance
The UKNEQAS for nuclear related antigens detects this particular pattern of staining on HEp-2 cells but no quantitative scheme exists for the ELISA. Negative distributions demonstrate 99% concordance. Positive distributions only show a concordance of 51% (range 46–58%). This suggests that there is a poor detection rate which may be due to poor tissue/cell choice, or the coexistence of other ANA which may mask the centromere pattern.

Commercial suppliers
IMF: See ANA. ELISA: L, SD.

ANTIPHOSPHOLIPID ANTIBODY (CARDIOLIPIN)

Antiphospholipid antibodies belong to a family of cross-reactive antibodies which react with negatively charged phospholipids, and which include anticardiolipin antibody. Cardiolipin is the antigen present in the tissue extract used for serological testing for syphilis. It has long been known that false positive results could be obtained persistently in syphilis testing and that

such false positivity could be associated with the development of SLE. False positive results could also be seen in SLE patients with acquired inhibitors of *in vitro* phospholipid dependent coagulation tests. More recently such antibodies have been more specifically associated with the primary antiphospholipid syndrome (APS) of thrombosis, recurrent fetal death and thrombocytopenia. Whilst the antibody in syphilis is authentic and directed against cardiolipin itself, it would now appear that the antibodies of APS are directed almost exclusively to phospholipid-binding plasma proteins, in particular, β_2-glycoprotein I (β_2GPI) and prothrombin, or complexes of these proteins with phospholipids. Hence this group of antibodies is best classified according to the method used to detect them.

Antiphospholipid antibody is detected by ELISA using cardiolipin, phosphatidyl serine or β_2GPI as the antigen whereas coagulation assays, e.g. activated partial thromboplastin time (aPTT) and dilute Russell viper venom time (dRVVT) detect antiphospholipid antibodies referred to as lupus anticoagulants. Both types of assay should be used in evaluating a patient for APS. However the coagulation assays do not lend themselves to large-scale screening and will not be covered by this review.

The intended use of antiphospholipid testing should be to assess the risk of thrombosis in patients with SLE or related disorders and for monitoring pregnancy in those patients at risk for fetal loss. Low or transient concentrations detected in infections and connective tissue diseases are not relevant to the diagnosis of antiphospholipid syndrome.

Reference material

To facilitate comparison of results between laboratories an international standard serum has been produced by the Anti-phospholipid Standardization Laboratory, University of Louisville, USA. The results are expressed in GPL units, MPL units or PL units for IgG, IgM and total antiphospholipid respectively. Many of the available commercial assays have been standardized against this serum preparation.

Reference preparation 90/656 is available from the UKNEQAS for autoimmune serology.

Assay types

Most laboratories are testing for the presence of cardiolipin antibodies using ELISA. IgG, IgM and IgA cardiolipin antibodies may be detected by ELISA assays, although only high positive IgG results are considered significant. Very little is known about the significance of IgM and IgA anti-cardiolipin antibodies.

It is important that β_2GPI is included in the reagents of ELISA kits.[82] There is some controversy over whether cardiolipin is an essential requirement. In some experimental systems antibodies could only be detected if both cardiolipin and β_2GPI were present, but other work has indicated that this was primarily methodological due to poor binding of β_2GPI to some microtitre plates in the absence of cardiolipin. If high-binding capacity plates are used it would appear that most if not all antibodies in the APS do recognize β_2GPI alone.[83]

Assay characteristics

A European study[84] highlights problems of anti-cardiolipin standardization. The multicentre study concluded that anti-cardiolipin testing standardization has not been achieved and that it was debatable whether standardization of such a heterogeneous system would indeed be possible. The problem is caused by the fact that cardiolipin antibodies represent a small subset of low affinity highly heterogeneous antibodies, whose binding *in vitro* may be influenced by several factors including the nature of the antibody (antibody isotype, avidity, cross-reactivity), the nature of the reference preparations used for standardization, and the design of the assay system. As IgG is the only class of antibody which can be measured with any degree of reproducibility, the measurement of IgM and IgA anti-cardiolipin antibodies is of doubtful value.

Impartial studies would suggest poor performance of greater than 20% coefficient of variation although kit manufacturers may claim better performance than this.

Quality assurance

Anti-cardiolipin antibody is included in the Autoimmune serology IIa scheme organized by the UKNEQAS. Serious inconsistencies have been revealed by the serum samples distributed by UKNEQAS.[85] Within-group coefficients of variation for the various commercial kits measuring only IgG cardiolipin antibody vary from 16–55% before calibration against RM90/656, and 19–36% after calibration. They are significantly dependent on the concentration of antibody present. Qualitative responses show less variation for the clinically significant antibody concentrations e.g. moderate to strong positive but are again significantly variable

around the clinical cut-off concentration. There is a significant lack of correlation between laboratories using the same kit and even more variation between commercial kits. In view of this, it is suggested that anti-cardiolipin be reported in a semi-quantitative format using four categories: negative, weak positive, moderate positive and strong positive.

Commercial suppliers
ELISA: A, BD, CG, CLS, IDS, I, L, N, SD, S.

COST OF ASSAYS

Precise costing of autoantibody testing is difficult. As with any immunoassay costs are highly variable depending on batch sizes, proportions of tests to standards and quality control samples, and of course how many of the components of the assay are purchased commercially and how many are produced within the laboratory. The following prices are a guideline only to the commercially available reagents when used with reasonable batch sizes of 20 or more test samples.

Agglutination assays and nephelometry for rheumatoid factor cost approximately £1 and £2 respectively, per patient sample. Pattern identification by fluorescent immunofluorescence using tissues or cells costs £1 per patient sample. The ELISA assays for single antigen specificities range in price from £1·50–£2·50 per patient sample and the Farr assay for DNA costs £2. The antibodies to ENA are usually investigated in panels (most commonly SSA, SSB, RNP and Sm). The cost of these ENA panels is approximately £20 by ELISA, £12 by immunodiffusion and £10 for immunoblotting per patient sample. These prices refer to reagent cost only, no consideration has been given to equipment, instruments etc., and it should be appreciated that the operator time will be very different depending on the method used.

CONCLUSION

This review has discussed the analytical aspects of current practises in autoimmune serology. Many of the techniques are subjective and labour intensive. Even now there are automated approaches to some autoantibody identification under development and near to full commercial production. These automated assays will almost certainly increase the general reagent cost but will also significantly reduce operator time and offer the potential for high throughput testing.

APPENDIX 1

Names and addresses, telephone and fax numbers of the commercial suppliers referred to in the text, together with their abbreviations.

A
Alpha Labs
40 Parham Drive
Eastleigh
Hants SO50 4NU
Tel: 01703-610911
Fax: 01703-643701

B
Behring Diagnostics UK Ltd
Walton Manor
Walton
Milton Keynes
Bucks MK7 7AJ
Tel: 01908-660000
Fax: 01908-680570

BD
Bio Diagnostics Ltd
Upton Industrial Estate
Rectory Road
Upton-upon-Severn
Worcestershire WR8 0XL
Tel: 01684-592262
Fax: 01684-592501

BK
Beckman Instruments (UK) Ltd
Oakley Court
Kingsmead Business Park
London Road
High Wycombe
Buckinghamshire HP11 1JU
Tel: 01494-429216
Fax: 01494-537642

BM
Boehringer Mannheim
Boehringer Mannheim House
Bell Lane
Lewes
East Sussex BN7 1LG
Tel: 01273-480444
Fax: 01273-480266

BS
The Binding Site Ltd
PO Box 4073
Birmingham B29 6AT
Tel: 0121-471-4197
Fax: 0121-472-6017

CG Cogent Diagnostics
Kings Building
West Mains Road
Edinburgh EH9 3JF
Tel: 0131-662-0067
Fax: 0131-662-1178

CLS Cambridge Life Sciences plc
Cambridgeshire Business Park
Angel Drove
Ely CB7 4DT
Tel: 01353-667034
Fax: 01353-664369

DPC DPC Biermann GmbH
DPL Division
Glyn Rhonwy
Llanberis
Caernarfon
Gwynedd LL55 4EL
Tel: 01286-871872
Fax: 01286-871794

E Eurogenetics UK Ltd
Unit 5
Kingsway Business Park
Oldfield Road
Hampton
Middlesex TW12 2HD
Tel: 0181-296-8800
Fax: 0181-296-9039

EP Europath Ltd
Highland Comport
Union Hill
Stratton
Bude
Cornwall EX23 9BL
Tel: 01288-353686
Fax: 01288-352866

IDS IDS Ltd/Inova
Boldon Business Park
Boldon
Tyne & Wear N35 9PD
Tel: 0191-519-0660
Fax: 0191-519-0760

I Incstar Ltd
Charles House
Toutley Road
Wokingham
Berkshire RG11 5QN
Tel: 01734-772693
Fax: 01734-792061

JJCD Johnson & Johnson Clinical Diagnostics Ltd
Mandeville House
62 The Broadway
Amersham
Buckinghamshire HP7 0HJ
Tel: 01494-431717
Fax: 01494-431165

L Launch Diagnostics
Ash House
Ash Road
New Ash Green
Longfield
Kent DA3 8JD
Tel: 01474-874426
Fax: 01474-872388

MD MAST Diagnostics Ltd
Mast House
Derby Road
Bootle
Merseyside L20 1EA
Tel: 0151-933-7277
Fax: 0151-944-1332

MN Metachem Diagnostics
29 Forrest Road
Piddington
Northamptonshire NN7 2DT
Tel: 01604-870370
Fax: 01604-870194

M MICROM UK Ltd
8 Thame Park Business Centre
Wenman Road
Thame OX9 3XA
Tel: 01844-213645
Fax: 01844-213644

N Nycomed (UK) Ltd
2111 Coventry Road
Sheldon
Birmingham B26 3EA
Tel: 0121-742-2444
Fax: 0121-722-2190

P Pharmacia Diagnostics
Pharmacia Ltd
Pharmacia House
Midsummer Boulevard
Milton Keynes MK9 3HP
Tel: 01908-603782
Fax: 01908-603799

Q Quadratech
PO Box 167
Epsom
Surrey KT17 2SB
Tel: 0181-786-7811
Fax: 0181-786-7822

RSR RSR Limited
Avenue Park
Croescadarn Close
Pentwyn
Cardiff CF2 7HE
Tel: 01222-732076
Fax: 01222-732704

S Sigma Chemical Company Ltd
Fancy Road
Poole
Dorset BH17 7NH
Tel: 01202-733114
Fax: 01202-715460

SD Shield Diagnostics Ltd
The Technology Park
Dundee DD2 1SW
Tel: 01382-561000
Fax: 01382-561056

SS Statens Seruminstitut
Artillerivej 5
2300 Kobenhaven S
DENMARK

U Unipath Ltd
Wade Road
Basingstoke
Hampshire RG24 0PW
Tel: 01256-841144
Fax: 01256-463388

W Murex Diagnostics Ltd
Central Road
Dartford
Kent DA1 5LR
Tel: 01322 277711
Fax: 01322 273288

APPENDIX 2

Names and addresses of the organizers of the relevant quality assurance schemes.

EQAS for Sperm Antibody
Mrs A Atkinson
Reproductive Laboratory Medicine
2nd Floor
St Mary's Hospital
Whitworth Park
Manchester M13 0JH

UK NEQAS for Autoimmune Serology
Department of Immunology
PO Box 894
Sheffield S5 7YT

APPENDIX 3

Names and addresses of the laboratories supplying reference material.

CLB
Department of Reagents
PO Box 9190
1006AD
Amsterdam
The Netherlands

AF/CDC ANA Laboratory
Immunology I-1202
A25
CDC
Atlanta
GA 30333
USA

National Institute for Biological Standards & Control (NIBSC)
Blanche Lane
South Mimms
Potters Bar
Hertfordshire EN6 3QG

PRU Procurement
Department of Immunology
PO Box 894
Sheffield S5 7YT

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