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Pitfalls in the performance and interpretation of clinical immunology tests

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ABSTRACT

A broad overview, with examples, of the potential pitfalls encountered in the clinical immunology laboratory is presented. Illustrative examples and case scenarios are provided from autoimmunity, immunochemistry and cellular immunology, looking at both technical and interpretative pitfalls.

In the UK, the performance of a clinical immunology laboratory is monitored both directly and indirectly. Examples include management systems monitored by accreditation schemes (eg, Clinical Pathology Accreditation (UK) Ltd) and analytical performance by external quality assurance schemes (eg, UK National External Quality Assurance Schemes (UK NEQAS)). Until recently, with the introduction of an interpretative online scheme by UK NEQAS, there had been little to examine the interpretation and reporting of immunology laboratory tests. It therefore seems timely to highlight some of the challenging areas for clinical immunologists.

Errors may be introduced owing to the quality of sample provided, quantity of analyte available or poor understanding of the assay leading to misinterpretation. Where appropriate, we illustrate these with short case scenarios or examples from practice.

SAMPLE QUALITY

Assays for functional complement

Three assays are routinely performed: haemolytic assays for the classical (CH50) or alternate (AP50) pathways and functional C1 inhibitor assays. These are all highly dependent on the integrity of the sample. Samples referred to a central specialist laboratory frequently arrive in less than ideal circumstances (eg, presenting to the laboratory unseparated or unfrozen more than 6 hours after taking). Storage of samples for more than 6 hours at room temperature may lead to complement activation *in vitro*.¹ Furthermore, it has been shown that activation takes place during coagulation.² Activation will in turn reduce the concentration of components available for the CH50 and AP50 assays, leading to falsely low results. Similar problems of poor sample integrity have been well documented to lead to misinterpretation of assays for, and misdiagnosis of, C1 inhibitor deficiency³ (see case scenario 1).

Case scenario 1

A 16-year-old man was referred to the immunology/allergy clinic with a history of recurrent urticaria/angioedema. The pattern of attacks

seemed entirely random, with some episodes occurring in the middle of the night, several hours after food and with no identifiable allergy triggers. There was no family history of note. Serum was sent by land post to a regional test centre, arriving at its destination 2 days later. Initial laboratory results included C3 1.07 g/l (reference range 0.8–2.1), C4 0.12 g/l (0.15–0.5), C1 inhibitor concentration 0.36 g/l (0.15–0.45), C1 inhibitor function <30% mean normal (70–130) and serum IgE 73 kU/l (reference range 1–133). After discussion with the immunology team, tests were repeated using fresh serum transported to the laboratory within 2 hours of venesection to exclude “decay artefact”. Results included C3 1.26 g/l, C4 0.13 g/l, C1 inhibitor antigen 0.23 g/l and C1 inhibitor function >100%.

Comment

These data were initially misreported as “raising the possibility of type II hereditary angioedema” (note: hereditary angioedema is not accompanied by urticaria, and is very rare—2 new cases/million per year). Decay artefact is by far the more likely explanation. The persistent borderline low C4 is a commonly found “red herring”, in this case most likely explained by partial inherited C4 deficiency. In the broader context other explanations for low C4 would need to be considered, eg immune complex disease, cryoglobulinaemia.

Cryoglobulins

Cryoglobulins are immunoglobulins that precipitate or gel at temperatures <37°C. There are several areas where caution is required in the investigation of cryoglobulins.

Firstly, when should the possibility of the presence of cryoglobulin be considered? There may be a failure to consider cryoglobulin as a cause of vasculitis, with the clinician focused on other autoimmune causes such as anti-neutrophil cytoplasm antibody (ANCA) related vasculitis or systemic lupus. The finding of a low serum C4 in the absence of other causes should prompt investigation. In a review of practice we found that of 15 type 1 and type 2 cryoglobulins all presented with serum C4 of ≤ 0.15 g/l (reference range 0.15–0.45 g/l) (unpublished data). Other anomalous laboratory findings, such as described below, should also lead to consideration of cryoglobulinaemia.

Secondly, a single analysis may be insufficient to exclude cryoglobulinaemia. Sample collection is critical and if clinical suspicion is high the test may need to be repeated on several occasions. False negative results owing to poor sample collection or

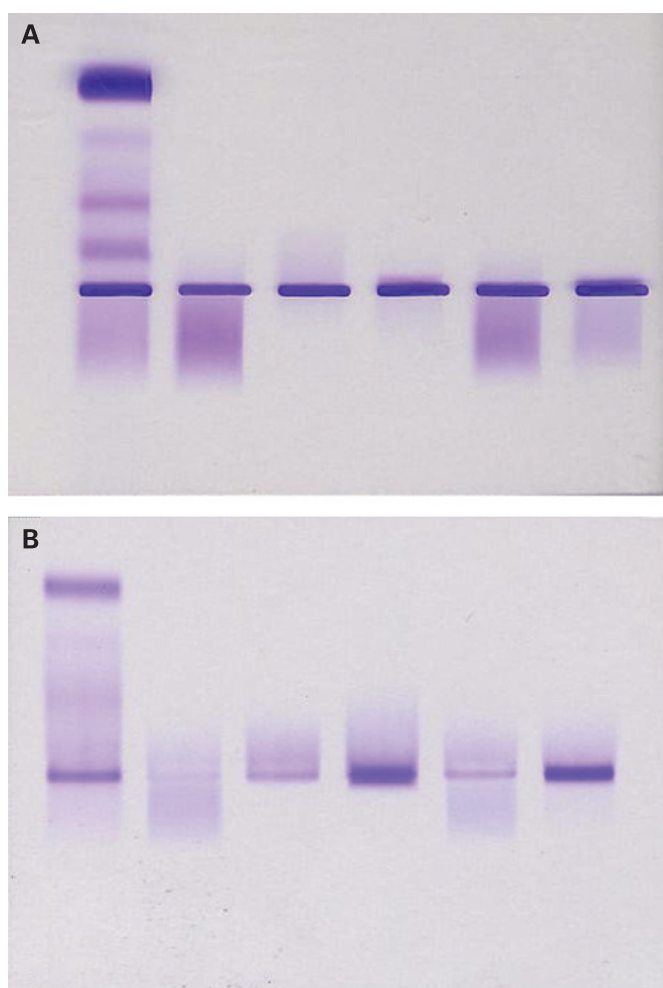


Figure 1 (A) Immunofixation of serum containing cryoglobulin unresolvable by immunofixation. Lanes are acid fixation, A, G, M, κ and λ . (B) Same serum after pretreatment with dithiothreitol showing presence of IgM- λ paraprotein.

separation, both of which must be at 37°C, are not uncommon. In our experience close liaison between laboratory and clinician is essential. A good quality sample is ensured if laboratory staff participate at the time of venesection and sample transfer by providing a 37°C container for transportation to the laboratory. Poor sample collection may lead to the cryoglobulin being lost with the blood clot. In extreme cases this could lead to a failure to identify the presence of a cryo-paraprotein and hence the associated lymphoproliferative disorder. Also of note, serum should be preferred to EDTA samples for the detection of cryoglobulin as cryofibrinogen may be found in the plasma of some normal individuals and thus may confound the analysis.

Thirdly, cryoglobulins may introduce artefacts into a variety of laboratory assays. These include problems with haematology analysers such as falsely high platelet counts or discrepancies between automated white count and manually estimated counts from the blood film. Immunofixation artefacts may also be seen, unresolved complexes at the origin being a particular problem. This may be resolved by preincubation with dithiothreitol (fig 1). Dithiothreitol reduces interchain disulphide bonds. This has a major effect on IgM, reducing it from the normal pentameric form to primarily monomeric form.⁴ In addition the action of dithiothreitol reduces cryoprecipitability.⁵

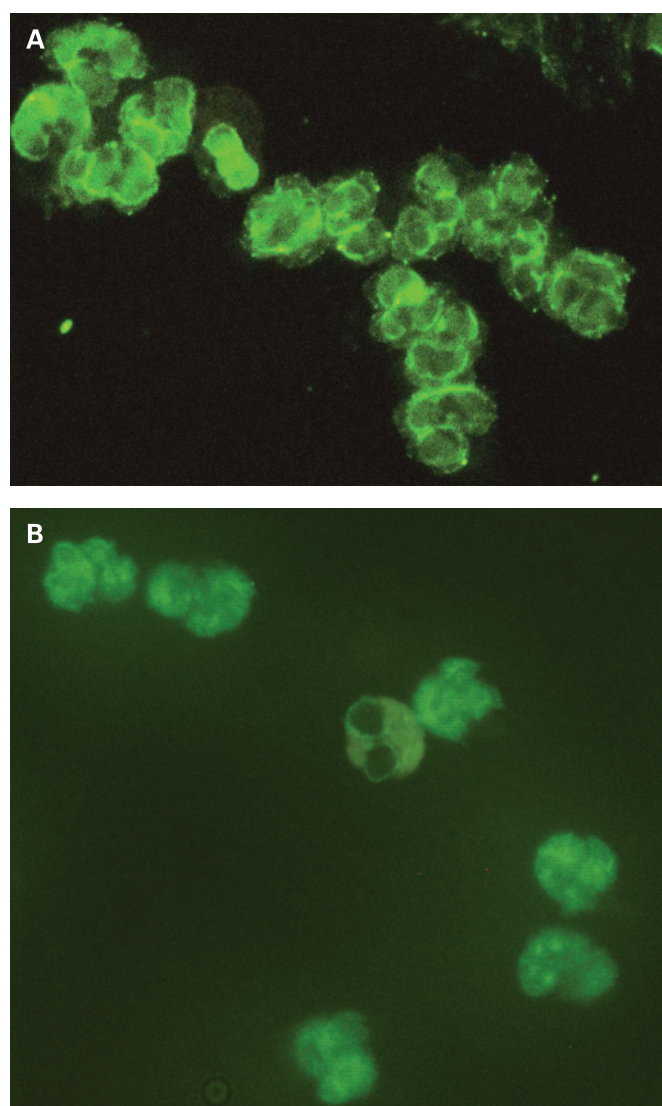


Figure 2 Interference in anti-neutrophil cytoplasm antibody (ANCA) assays by anti-nuclear antibody (ANA). (A) Perinuclear ANA from patient with systemic lupus erythematosus, raised anti-dsDNA and no evidence of current vasculitis. MPO-ANCA and PR3-ANCA were negative. Stain was completely abrogated on formalin fixed neutrophils. (B) High titre ANA obscuring underlying MPO-ANCA from patient with rapidly progressive glomerulonephritis (central eosinophil shows weak peripheral stain only in nucleus).

ERRORS OF MISINTERPRETATION

Indirect immunofluorescence is used for the detection of many autoantibodies. The interpretation of staining patterns relies heavily on the skill and experience of the reader. However, in addition there are specific technical concerns.

Anti-neutrophil cytoplasm antibody (ANCA)

Despite the introduction of a consensus document⁶ some patterns still provide a challenge. In particular, on ethanol fixed neutrophils anti-nuclear antibodies (ANA) may mimic ANCA (fig 2A) or mask an underlying ANCA-related specificity (fig 2B). Not all laboratories offer specific ELISA tests for myeloperoxidase (MPO) and proteinase 3 (PR3) antibodies. Historically, formalin fixed neutrophils were used to distinguish ANCA from ANA⁷ but this was not always entirely reliable⁸ and is no longer recommended. We would reiterate the advice given in the

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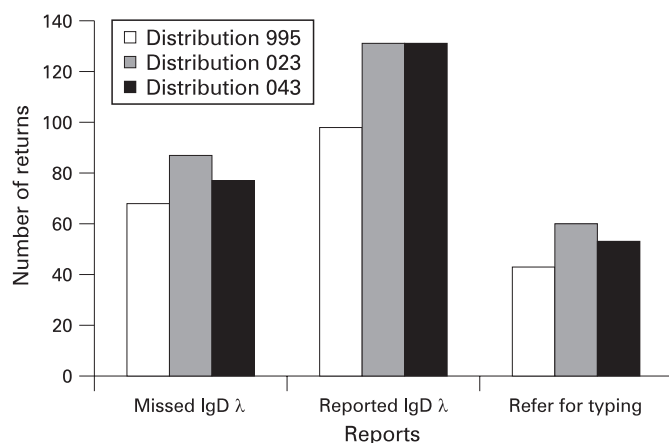


Figure 3 Returns from three distributions of IgD-λ paraprotein showing that approximately one-third of all laboratories failed to consider the presence of IgD on each occasion (reproduced from the UK NEQAS Annual Report for monoclonal proteins 2004, with permission. www.immqas.org.uk/2005_annual_report/report.pdf).

consensus document that any serum giving an immunofluorescence pattern seen on ethanol fixed neutrophils demonstrating ANCA, any other cytoplasmic stain or homogenous or perinuclear ANA should be investigated for antibodies to PR3 and MPO.⁶

IgG anti-desmosome antibodies (pemphigus investigation)

The presence of anti-desmosome antibodies is characteristic of pemphigus (vulgaris and foliaceus). A variety of substrates, with different sensitivities for detection of anti-desmosome (also formerly called anti-intercellular substance), are in use, with monkey oesophagus being the most popular in the UK. Two separate problems may be associated with the use of this substrate.

Firstly, the endogenous (background) monkey IgG in the tissue section, as well as the patient's autoantibody, may react with anti-“human” IgG antisera (because cross reaction with primate IgG is not uncommon). Increased background staining of the monkey IgG in the tissue can obscure true weak anti-desmosome antibodies derived from the patient. For this reason we advocate the use of antisera that have been affinity absorbed against monkey IgG. Note that background IgG when using human substrates (eg, human small bowel for the detection of anti-enterocyte antibody) is potentially a problem for direct immunofluorescence techniques also, with endogenous human IgG increasing the background.

Secondly, antibodies to A or B blood group antigens may react with the equivalent tissue antigens to give a false positive pemphigus-like pattern,^{9, 10} mimicking the anti-desmosome pattern. A and B antigens have been shown to be expressed on the spinous cells of non-keratinised oral mucosa.¹¹ Positive sera may be checked for false positive reactions by prior absorption or use of commercially available reagents, which will negate the reaction.

Serum protein electrophoresis

The primary use of this assay is for the detection of monoclonal proteins. However, analysis is initiated in a variety of circumstances and other important diagnoses must not be overlooked (eg, immunodeficiency associated with

pan-hypogammaglobulinaemia, or HIV suggested by huge levels of polyclonal human immunoglobulin).

When a paraprotein is detected it is standard practice to type the paraprotein by immunofixation for both heavy (α , γ , μ) and light (κ , λ) chains. In the absence of heavy chain reactivity (α , γ or μ chain), detection of clonal light chain suggests the possibility of Bence Jones myeloma (20% of myeloma presentations). However, a small percentage will be either IgD or IgE myeloma and this should be excluded by immunofixation with appropriate heavy chain antiserum (δ , ϵ). Experience in the UK NEQAS is that this is overlooked by a large minority of participating laboratories (fig 3).

The absence of an obvious paraprotein does not exclude its presence. Paraproteins running in the β region or β - γ region may be overlooked on serum protein electrophoresis (fig 4, lanes 1 and 3). The appearance of hypogammaglobulinaemia (fig 4, lane 5) should prompt investigation for monoclonal free light chains. These may migrate with other proteins and so not appear as a separate band. The presence of an unexpected band does not necessarily confirm malignancy; see, for example, fig 4, lane 6, which shows fibrinogen in normal plasma. Other common patterns are the split β band seen in old samples, owing to C3 degradation to C3c, and the split α -2 band seen in some persons with haptoglobin variants.

TOO MUCH, TOO LITTLE OR THE WRONG SORT

Specific IgE assays

False positive “specific” IgE results may be seen in the context of persons with high total serum IgE (case scenario 2). With older manual systems such as the radio-allergosorbent test (RAST) it was possible to introduce extra washing steps which would reduce this problem. The newer closed systems are also prone to this error, albeit at higher concentrations than was the case with RAST. A series of positive specific IgE results, with no negative results should be viewed with suspicion (this is particularly true of the patient with atopic dermatitis where false positive results are common¹²).

Case scenario 2

A 33-year-old man presented to the allergy clinic with a rash, convinced that he had food allergy. The clinical history however was unconvincing. Atypical symptoms (headaches and tiredness) were often experienced a day or two after eating, and foods under suspicion could sometimes be eaten in smaller amounts without symptoms. On examination, the patient did have atopic eczema. His serum IgE was 4654 kU/l (1–133). Specific IgE to hens' egg white was 0.98 kU A/l (0–0.35) (grade 2), to cows' milk 0.51 kU A/l (grade 1) and to wheat 2.19 kU A/l (grade 2), although he had no history of immediate hypersensitivity to any of these allergens.

Comment

When the regional assay centre performed additional tests to clinically irrelevant unrelated allergens (bee venom and penicillin V), similar low grade “false” positive results were obtained. The results suggested non-specific, weak false positive results due to high background IgE, to be interpreted with caution in the context of the clinical history.

Prozone and antigen excess

The prozone phenomenon has been recognised for many years, especially in agglutination systems where extremely high titre antibody coats the particle without cross linking to adjacent

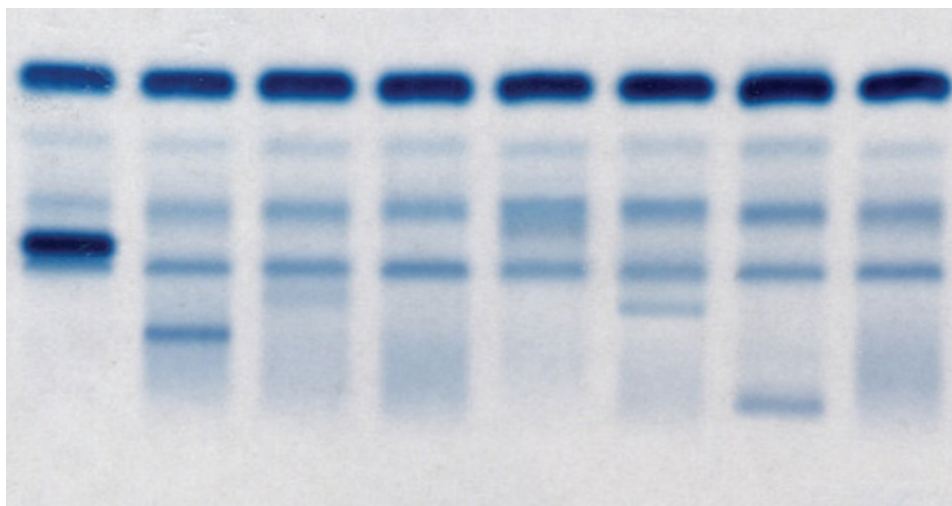


Figure 4 Serum protein electrophoresis. From left to right, the following were demonstrated by immunofixation: (1) IgG- κ + free κ light chains (both β region); (2) IgM- λ ; (3) IgA- κ (β - γ region); (4) normal; (5) hypogammaglobulinaemia; (6) normal plasma (note fibrinogen in slow γ); (7) IgG- κ ; (8) normal.

antigen-coated particles, generating a false negative result. This may be corrected by dilution of the serum. Examples include anti-thyroid antigens (IgG class antibodies) (fig 5) and rheumatoid factor (IgM class antibodies). Thus screening at a single dilution may yield false negative results.

Essentially the same phenomenon is present in fluid phase systems (nephelometry and turbidimetry), only here the phenomenon is seen in reverse (ie, due to antigen excess). One recent assay to suffer from this pitfall is the FreeLite Assay (Binding Site, Birmingham, UK). The assay has proved to be of clinical utility in the evaluation of patients with AL amyloid and non/poorly secreting myeloma.¹³ However, its extended use into more overt disease such as light chain myeloma has highlighted some deficiencies in the system, with falsely low results owing to missed antigen excess (as seen in some UK NEQAS distributions) or failure to recognise some paraproteins¹⁴ and overestimation owing to non-linearity of the assay (case scenario 3). Furthermore these phenomena may depend on the platform used for the analysis (unpublished data). A further problem regarding comparability of the FreeLite assay between platforms has recently come to light. Pattenden *et al* showed that reference ranges differ according to the platform used¹⁵ and that local, analyser-specific reference ranges should be determined.

Case scenario 3

An 81-year-old man presented with back pain and was found to be anaemic with a raised erythrocyte sedimentation rate. A myeloma was suspected. Serum IgG, IgA and IgM were all reduced, but despite this apparent immune paresis no paraprotein was found on routine electrophoresis. Immunofixation showed a barely detectable (κ) band, too small to quantify by standard protein electrophoresis (ie, <1 g/l). Serum FreeLite

quantified the serum free κ light chains at 13.7 g/l. Urine analysis revealed a κ Bence Jones protein at 2 g/l on an early morning urine (although 24 hour output would be more useful for monitoring). Bone marrow aspirate and trephine confirmed myeloma.

Comment

Serum light chain 14 g/l would lead to a very clear dense band (not seen here). The FreeLite assay clearly overestimated the free light chain concentration in the serum of this patient. In EQA schemes, this very useful but imperfect assay can give inter-laboratory coefficients of variation as high as 60%. The affinity purified antibodies used in this assay do not give reliable results in 100% of monoclonals tested.

Antibody avidity and affinity

Assay characteristics, such as pH and ionic strength, will have significant effects on antibody-antigen reactions. Gel precipitation based assays, for example for antibodies to extractable nuclear antigens (eg, Sm antigen), and radio-precipitation assays for anti-double stranded (ds) DNA, had low sensitivity but high disease specificity for systemic lupus erythematosus (SLE). Over recent years the trend has been towards the use of higher throughput ELISA systems, with improved sensitivity but often with reduced specificity owing to the detection of low affinity antibodies not detected in the older assays.¹⁶ This reduces the positive predictive value of the test and may lead to over-interpretation of positive results¹⁷ (case scenario 4). Modified ELISAs designed to detect only high avidity anti-ds-DNA have been developed, but it is not clear that these offer any significant advantage.¹⁸

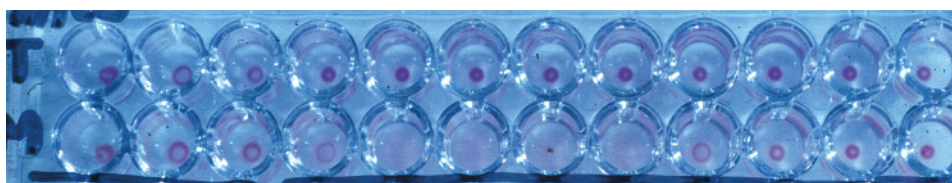


Figure 5 Example of prozone for anti-thyroid antibody investigation. Upper row, anti-thyroglobulin (negative); lower row, anti-thyroid microsome (wells 1-4 prozone, 5-8 positive) (Serodia-ATG and Serodia-AMC, Fujirebio).

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Case scenario 4

A 63-year-old man with arthralgia was found to have high titre anti-ds-DNA antibody despite an initial negative ANA screen at a serum dilution of 1/80 using rat liver sections as substrate. However, the consultant rheumatologist regarded osteoarthritis as the likely diagnosis on the basis of clinical examination and radiological investigation. The regional immunology laboratory was asked to perform additional tests. ANA was negative on a human epithelial cell (HEp2) substrate. Ds-DNA antibodies were positive in a similar but different ELISA assay; indirect immunofluorescence testing using *Crithidia luciliae* as substrate was negative.

Comment

The unicellular protozoan *Crithidia luciliae* contains an extra-nuclear package (Kinetoplast) entirely composed of ds-DNA. It is a useful and reliable substrate for testing/confirming true high affinity ds-DNA antibodies. In this case the laboratory reported the likelihood of low affinity ds-DNA antibody of uncertain significance. This accorded with the rheumatology opinion and the fact that the patient did not fulfil the ARA (American Rheumatology Association) criteria for SLE.

TIMING IS OF THE ESSENCE

In the investigation of anaphylaxis, mast cell tryptase (MCT) has proved to be a very valuable tool. In our experience there are several areas where the unwary can be misled.

Following an anaphylactic episode, MCT is released and passes into the bloodstream within 30–60 minutes. A sample taken before this time may thus falsely point against anaphylaxis. MCT levels peak around 60–120 minutes, and the half life is approximately 2 hours.¹⁹ Samples are less useful after 6 hours post the event.²⁰ MCT should normalise by 24 hours.

In many cases following resuscitation, a colloid infusion may lead to haemodilution, and again false negative interpretation may ensue. We advocate measuring other bystander serum proteins simultaneously to get some gauge on the extent of possible serum dilution (our practice includes IgG, C3 and C4).

A proportion of patients with underlying myeloproliferation disorders or mastocytosis have high baseline levels of MCT and a high one-off sample at one hour could be misinterpreted as support for anaphylaxis. As a minimum, a positive result should always trigger a request for a baseline sample post recovery (ie, at least 24 hours after the triggering event).

An additional area where confusion arises is with the stability of MCT in vitro versus in vivo. The in vivo half life is about 2 hours, which is why sample timing is critical. However, once sampled into a test tube, MCT is a stable antigen which degrades relatively slowly. Neither rapid transport nor frozen samples are necessary. MCT can also be useful in the investigation of anaphylaxis post-mortem. Note that in this

latter scenario blood should not be taken by heart stab as the heart is rich in mast cells²¹ that slowly degranulate after death, often resulting in higher levels than seen by femoral vessel sampling.²²

Comment

We advise that, ideally, three separate (timed) samples taken at 1 hour, 3 hours and 24 hours post reaction are made available for testing.

INTERNAL CONSISTENCY OR “MAKING THINGS ADD UP”

There are few controls available in some fields of immunological analysis. Flow cytometry is a particularly difficult area in this respect. Case scenarios 5, 6 and 7 illustrate the importance of internally consistent results.

Cases scenarios 5, 6 and 7

Three paediatric cases, all suspected of immunodeficiency, are presented. Diagnostic tests included analysis of lymphocyte sub-populations by flow cytometry. Results are summarised in table 1.

Case scenario 5

A 3-month-old boy was admitted with fever and breathing difficulty. His condition rapidly deteriorated, and *Pneumocystis carinii* infection was identified. Family history included a deceased maternal uncle who had died of pneumonia in infancy 25 years previously (ie, suggestive of an inherited X-linked condition). Lymphocyte subset analysis (see table 1) showed lymphocytopenia, strikingly low T cell numbers (effectively a pan T lymphocytopenia), but elevated B cell numbers. The clinical picture, family history and lymphocyte subset results all pointed to X-linked inherited severe combined immunodeficiency. The immunophenotype (T– B+ NK–) was suggestive of interleukin γ chain deficiency; this diagnosis was confirmed genetically.

Comment

Importantly, all the numerical sub-populations add up as expected. Thus, the total lymphocyte count should be the sum of the T, B, and NK counts. Also the total CD3 count should be the sum of the CD4 and CD8 counts. Hence, the results in this case are all believable, even though the lymphocyte pattern is markedly abnormal.

Case scenario 6

This was a 5-year-old boy with weight loss, failure to thrive, diarrhoea, and odd T cell rich cutaneous lesions. Lymphocyte subset analysis did not “add up”. Thus the sum (2.0) of the T (CD4 plus CD8), B, and NK counts (0.6, 0.2, 0.2) leaves unexplained a “gap” when compared to the total lymphocyte

Table 1 Lymphocyte subset analysis in three cases of suspected immunodeficiency

Antigen (cell type) evaluated	Case 5 cells $\times 10^9/l$ (age-related reference range)	Case 6 cells $\times 10^9/l$ (age-related reference range)	Case 7 cells $\times 10^9/l$ (age-related reference range)
Total lymphocytes	1.8 (2.7–5.4)	2.0 (2.9–5.1)	3.9 (2.9–5.1)
CD3 (T cell)	0.2 (1.7–3.6)	1.6 (1.8–3.0)	2.4 (1.8–3.0)
CD4 (T cell)	0.1 (1.7–2.8)	0.4 (1.0–1.8)	0 (1.0–1.8)
CD8 (T cell)	0.1 (0.8–1.2)	0.2 (0.8–1.5)	0.9 (0.8–1.5)
CD19 (B cell)	1.5 (0.5–1.5)	0.2 (0.7–1.3)	1.0 (0.7–1.3)
CD16 (NK cell)	0.1 (0.3–0.7)	0.2 (0.2–0.6)	0.5 (0.2–0.6)

count (2.0). Further analysis showed that an additional abnormal population (1.0) of γ/δ (as opposed to α/β) T cells were present in the circulation.

Comment

Normally, the majority (>95%) of blood T lymphocytes are of the CD3 positive, α/β antigen receptor type. Here, >50% were CD3+ but CD4 and CD8 negative, with the less usual γ/δ antigen receptor chain type (normally <5%). This rare finding can be seen in certain gut and other disorders. When the numbers do not “add up”, repeat confirmatory testing and further appropriate immunological investigations must be undertaken. For example, where CD19+ B lymphocytes seem to be absent, alternative B cell surface markers could be tested, including CD20.

Case scenario 7

This 6-year-old girl gave a history of recurrent sore throats. However, she was thriving on the 60th centile for weight, with no hospital admissions and no relevant family history. Multiple throat swabs were normal. Immunoglobulin levels were normal and serological responses to test vaccines all normal. Lymphocyte subset analysis showed nil CD4+ lymphocytes. This finding was discussed with the laboratory. Initially HIV or idiopathic CD4 cytopenia were suggested as possible diagnoses. Repeat samples were referred 2 weeks later to a regional immunophenotyping laboratory where analysis showed completely normal values for the CD4 and all other subsets.

Comment

The results quoted in table 1 do not add up. The CD3 count should be the sum of the CD4 and CD8 counts. This was not so for case scenario 7. The likely explanation is that the anti-CD4 test reagent was either faulty or omitted from the assay first time around. The original results should not have got past the authorisation stage, where repeat testing should have been advised.

OVER-INTERPRETATION OR “NEVER SAY NEVER”

The strength of many pathology tests is their high negative predictive value, that is, they have the ability to exclude disease; for example, a negative anti-nuclear antibody test makes systemic lupus very unlikely. Conversely, in a minority of tests, there is a high positive predictive value where a positive result has a high probability of confirming the presence of disease; for example, IgA anti-endomysium is a good marker of occult coeliac disease. However, the predictive values are rarely sufficiently high to be definitive regarding the diagnosis. Below we give just two examples where it is wise not to overstate the strength of the data.

False dictum 1: “Negative serum specific IgE to peanut excludes peanut allergy”

While peanut allergy may well be unlikely when IgE specific peanut antibody is absent, food allergy is best diagnosed on the basis of clinical history, and can only be definitively excluded (where clinically appropriate and at risk) by inpatient challenge testing.

False dictum 2: “Negative IgA anti-tissue transglutaminase antibody excludes coeliac disease”

IgA anti-endomysium and IgA anti-tissue transglutaminase antibodies have high sensitivity and specificity for the diagnosis

Take-home messages

- ▶ For functional assays (complement, lymphocyte function) sample integrity is crucial. Cryoglobulins require warm separated samples.
- ▶ Indirect immunofluorescence assays are subjective and subject to interference from both auto- and allo-antibodies.
- ▶ Not all extra bands on serum protein electrophoresis are paraproteins.
- ▶ Both antigen and antibody excess remain potential confounders in immunochemical assays.
- ▶ The regional immunology laboratory is a useful point of reference.

of coeliac disease.^{23 24} However, 1–2% of patients with coeliac disease have IgA deficiency and will be negative in the test.²⁵ In cases with high clinical suspicion an IgG based assay should be used, although these in turn must be treated with caution as specificity is lower. A second trap for the unwary is that, in our experience, increasing numbers of patients present on a self-imposed gluten-free diet. If this has been successful the autoantibodies may have disappeared before the consultation and blood testing have occurred.

FINAL COMMENT

Captain Edward Murphy was right. If a thing can go wrong, it will.

Competing interests: None declared.

Patient consent: Patient consent not required. All clinical scenarios are fictional in terms of age, gender etc, but based around real lab data (from anonymised patients).

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