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Laboratory diagnosis of rubella: past, present and future

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INTRODUCTION

Fifty years ago in New South Wales the late Sir Norman Gregg [1] described congenital cataracts in 78 babies, 67 of whose mothers had had clinical rubella in early pregnancy; he concluded that the disease in the mother caused the abnormality in the baby. Gregg [1–3] and Swan [4, 5] and their colleagues reported that deafness, heart disease and microcephaly were also major components of the congenital rubella syndrome. The need to prevent this tragic outcome stimulated intensive work on laboratory diagnosis and vaccine development, leading to the isolation of rubella virus in 1962 and then to methods for antibody detection. These complementary advances established the two traditional pillars of virological diagnosis and opened the way to immunization, with the result that some countries are now on the verge of eliminating a disease which for over 100 years was regarded as no more than a mild and harmless exanthem of childhood.

Accurate laboratory diagnosis of past or recent rubella is essential, not only for the individual patient but also for epidemiological reasons. Only about half the illnesses that are diagnosed clinically as acute rubella are actually caused by rubella virus, and an unknown number of rubella infections are symptomless but no less dangerous. For the woman with a rash in early pregnancy, therefore, accurate diagnosis is vital if unnecessary abortion is to be avoided since only rubella, so far as we know, is teratogenic. In a wider context, all such rashes must be investigated before calculating the frequency of fetal abnormality since otherwise the inclusion of non-rubella rashes will lead to underestimation of the risk. Laboratory diagnosis of congenital infection is needed both for the benefit of the individual child and for assessing the separate risks of fetal infection and consequent damage. Similarly, tests for immunity are required not only for individuals such as adult women seeking vaccination and pregnant women who come into contact with rubella, but also for designing a vaccination programme and monitoring its impact. In short, virology and epidemiology must go hand-in-hand.

VIRUS ISOLATION

In 1962 Weller and Neva [6] at the Harvard School of Public Health and Parkman, Buescher and Artenstein [7] at the Walter Reed Army Institute of Research independently isolated filterable agents from blood, urine or throat washings from young adults with clinical rubella. Weller and Neva described a cytopathic effect (CPE) in cultures of primary human amnion, with the development of amoeboid cells containing aggregated nuclear material and

refractile inclusions. However, the CPE took 17–37 days to appear and was not always easy to identify. Neva and Weller [8] reported that the changes were more obvious in later passages, but other workers experienced difficulty with this system. Parkman and his colleagues reported that rubella virus, although not visibly cytopathic, rendered primary African green monkey kidney (GMK) cells resistant to lytic infection by echovirus type 11. The cells were tested for interference after 7–14 days but sometimes required more than one blind passage because cells from second and third passages resisted progressively greater challenge doses. Several subcultures were necessary to produce sufficient virus for use in neutralization tests. Owing to the need for challenge, repeated examination of the same tube was impossible. The agents isolated by both groups of workers could be serially transmitted, were antigenically similar and were neutralized by convalescent sera from patients and experimentally infected animals [9, 10]. Other combinations of cell and challenge virus have been found to show exclusion by rubella, but suitable combinations are unpredictable and must be discovered by trial and error. In America, after the epidemic of 1964, the interference system was used to isolate virus from the throats of babies with congenital infection and also from amniotic fluid, placentas and a variety of fetal tissues after rubella in early pregnancy (reviewed by Alford and Kanich [11]). This work, combined with histological studies [12], led to the recognition that congenital rubella was not merely a syndrome of abnormalities resulting from a single blow at a crucial stage of pregnancy but a chronic multisystem infection which persisted for months after birth. The clinical spectrum was found to be wide, ranging from asymptomatic infection to severe disease of which malformations were only a part.

In good hands the interference system worked well, but it remained a cumbersome method in which the use of primary monkey kidney cells introduced the possibility of unknown effects by latent viruses. In 1963 McCarthy, Taylor-Robinson and Pillinger [13] reported that rubella virus caused a CPE in the RK13 continuous line of rabbit kidney cells. Further experience and changes in technique enabled them to recognize characteristic foci which were visible at low magnification after 3 days and consisted of refractile debris surrounded by a halo of thinned-out hyperplastic cells [14, 15]. The use of a continuous cell line had clear advantages, and the focal nature of the CPE offered a straightforward way of titrating both virus and antibody. RK13 cells therefore came into common use in the UK and led to the isolation of virus from the throats of patients with acute rubella [16] and from embryos infected during pregnancy [17, 18]. Unfortunately the method is not without problems because the CPE depends greatly on the culture medium and the state of the cells. The medium must be at the correct pH and must contain suitable batch-tested serum; the cells can appear to lose their properties, necessitating a return to low passage stock from liquid nitrogen; true foci are sometimes evanescent and may be missed if the cultures are not inspected frequently. Confirmation by neutralization is necessary because similar appearances may be produced by other viruses, particularly parainfluenza, or by non-specific degeneration.

It is now clear that rubella virus causes non-lytic infection in a variety of cells from different species, but without visible change, which perhaps accounts for the failure of early attempts at isolation. Specific antigens are nevertheless expressed

and can be detected immunologically. For example Schmidt and colleagues [19] and Woods and colleagues [20], reported that antigen could be demonstrated in RK13 cells by indirect immunofluorescence, using either hyperimmune rabbit serum [19] or convalescent human serum [20] followed by the appropriate anti-species fluorescein conjugate. The use of human serum can reveal irrelevant antigen–antibody reactions, but in these experiments rubella specificity was checked by blocking with hyperimmune serum prepared in rabbits. In fact no single cell type under constant cultural conditions satisfies the separate requirements of sensitivity, CPE, antigen expression and yield of infectious virus; it is better to isolate the virus in one line and then subculture to another for evidence of infection. In recent work virus was isolated from fetal tissues by inoculation into tube cultures of vero cells to produce a high titre of infectious virus, followed by passage to coverslip cultures of BHK21 cells; no recognizable CPE appeared in either cell line but intracellular antigen was detected in the BHK cells by immunofluorescence using human serum and fluorescein-labelled anti-human globulin [21]. The risk of detecting irrelevant antibodies was reduced by using serum from a child aged one year with congenital rubella, and specificity was additionally checked by repeating the staining with monoclonal rubella antibody and fluorescein-labelled anti-mouse globulin. This technique gives an unmistakable all-or-none result but is no less laborious than previous methods.

Virus isolation was the key that enabled early workers to study the natural history of acute and congenital rubella, but in diagnostic work it has now been largely replaced by serological methods which are quicker, more reliable and not limited to the brief period of virus excretion.

TESTS FOR ANTIBODY

Diagnosis of acquired rubella

The isolation of rubella virus led to tests for neutralizing antibody using either the interference system or, later, RK13 cells as indicators. Unfortunately the procedure was cumbersome and insensitive, and titres were low. In 1965 Sever and colleagues [22] introduced a complement fixation test (CFT), using antigen extracted from RK13 cells. The simple and familiar CFT technique was attractive, and the relatively slow development of CF antibody encouraged the hope that the titre might continue to rise after neutralizing antibody had reached its peak. In practice the CFT was disappointing because it depended greatly on the quality of the antigen, particularly the proportions of soluble and virion components. Antibody titres were variable and often declined to undetectable levels in 2–5 years. Both methods were eclipsed by haemagglutination-inhibition (HI) which had been used for many years in arbovirus work and was introduced to the rubella scene by Stewart and colleagues in 1967 [23]. The HI test, using avian erythrocytes, showed good correlation with neutralization but was simpler and more sensitive; titres were higher and antibody could be detected in adults who had had rubella in childhood. HI detected all classes of antibody and could therefore be used to measure specific IgM in serum fractions (see below). It did not require live virus but used an antigen which could be freeze dried and which soon became available commercially.

The HI test quickly became the cornerstone of rubella serology, but it has some drawbacks. The first of these is its vulnerability to non-specific inhibitors of haemagglutination, mainly low density beta-lipoproteins, which must be removed from each serum before testing. Two procedures are in current use for this purpose, namely treatment with kaolin or with a mixture of heparin and manganous chloride. Both methods occasionally fail, particularly the latter. Other techniques employing dextran or rivanol have been described but have no special advantages. Kaolin is generally preferred because it is simpler and more effective than heparin/manganous chloride which has the extra disadvantage of occasionally causing haemagglutination. A second difficulty with the HI test is the variation which occurs, both within and between laboratories. This problem was investigated in 1978 by a Committee of the Public Health Laboratory Service (PHLS) which concluded that variation between laboratories could be reduced by using a standardized method (described in their report) and comparing the test serum with a control of known potency in international units (i.u.) per ml [24]. In 1985 Murray, Stanton and Gardner [25] showed that much of the variation was due to the presence of gelatin in the special veronal buffer. They recommended using ordinary CFT buffer with the addition of 0·2 % albumen which gave higher haemagglutinin titres, more consistent HI results and clearer end-points. These improvements occurred regardless of the erythrocyte species, but the latter can also cause problems: chick cells vary in quality according to their source, and pigeon cells are sensitive to non-specific agglutinins which must first be absorbed from human serum. Consistency can be improved by using trypsinized human group O cells, provided that these can be obtained regularly from a reliable source. In practice a moderate degree of variation does not interfere with the diagnosis of acute infection because the demonstration of a rise in titre is more important than the antibody content. No amount of standardization can override the principle that all sera from a patient with suspected infection must be tested together in one laboratory.

Despite its problems the HI test is still the most widely used procedure for titrating antibody for diagnostic purposes, and it remains the method with which others are traditionally compared. Other titration techniques are used in a few laboratories for special purposes. Indirect immunofluorescence (IF), using a monolayer of infected cells as a substrate, was introduced for rubella antibody detection by Brown and colleagues [26] and later refined to identify antibodies of separate immunoglobulin classes [27]. Briefly, infected monolayers are treated first with patient's serum and then with class-specific antibody labelled with fluorescein. IF has several advantages: first, the antigen–antibody reaction is not confined to haemagglutinin; secondly, the intracellular display of this reaction helps to ensure specificity; thirdly, IF is a test of primary binding, independent of secondary phenomena such as neutralization, complement-fixation and haemolysis. These advantages, coupled with sensitivity (given a good optical system) enable low titres of IgG antibody to be detected with confidence. IF can therefore be used to investigate apparent failure of vaccination, which is often due to a low level of pre-existing immunity, and to distinguish between reinfection and primary asymptomatic infection in women who present with symptomless seroconversion after contact. IF will also detect IgG antibody in children with congenital rubella in whom the HI titre has become low or even negative [28].

However, IF has obvious disadvantages: considerable background work is involved in making preparations for staining, a suitable microscope is required and finally the result must be read subjectively. These drawbacks restrict IF to the investigation of special problems in a few laboratories.

Indirect enzyme-linked immunoassay (ELISA), like IF, is a sensitive test of primary binding which will detect low titres of IgG antibody [29]. Methods and applications for rubella work have been reviewed by Morgan-Capner and colleagues [30]. IgG antibody can be titrated in the traditional way by testing doubling dilutions of serum. Alternatively, IgG can be estimated from a single dilution, either by measuring the absorbance or, preferably, by comparing the test serum with a range of standards. Expertise, careful timing and stringent quality controls are necessary, particularly if a chromogenic substrate is used. At present ELISA seems unlikely to replace HI for the diagnosis of acute rubella, but this situation could change if better defined antigens were to become available or if enhanced luminescence were to replace chromogenesis, as, for example, in the 'Amerlite' system (Amersham International plc).

Seroconversion, or at least a significant rise in antibody titre, is the best confirmation of acute rubella, but the need to establish or exclude the diagnosis with inappropriately timed specimens led to a large body of work on the detection of specific IgM, which became recognized as a marker of recent infection. Early work was directed at titrating IgG and IgM antibodies separately after fractionation on a sucrose gradient [31] or by gel filtration [32]. Both fractionation procedures are reliable if used regularly (as they still are in some laboratories) but their cumbersome nature limits their use, so that judgement is needed in deciding which specimens to test. Indirect IF using labelled anti-IgM was introduced in an attempt to circumvent these limitations [33] but never became sufficiently reliable for routine diagnostic use. Analogous methods using an enzyme [34] or a radioactive [35] label have been developed but all such antiglobulin techniques are subject to two main sources of error when whole serum is tested, namely that IgM detection may be depressed by competition from IgG and that false positive results may occur if IgM with anti-IgG activity is present.

A major improvement came with the introduction of the antibody class capture assay based on the work of Diment and Pepys [36]. Patient's serum is added to a solid surface (bead or well) coated with anti-IgM; some of the patient's IgM is immobilized, regardless of specificity, and the specific component is then detected by the addition of antigen (haemagglutinin) which is in turn recognized by antibody labelled with an enzyme or a radioactive isotope. The radio-label is slightly more sensitive but the enzyme label has a longer working life and is not hazardous to prepare. Since the capture assay measures the proportion of the total IgM that is specific, it is largely independent of concentration as long as the total IgM is sufficient to saturate the binding sites on the solid phase. Specific IgM can thus be assayed from a single dilution by comparing the test serum with a set of standards made from mixtures of IgM-positive and antibody-negative serum. As these standards contain graded proportions of specific IgM, regardless of titre, they cannot be used in an indirect (antiglobulin) type of test.

The capture assay, requiring only 5 μl of serum, is more sensitive and reliable than previous methods. Theoretically, it could be affected by preformed complexes of IgG antibody with IgM, but in practice it is remarkably resistant to the effects

of rheumatoid factor. It virtually revolutionized the detection of IgM antibody and has been applied to the diagnosis of infection with a wide range of different viruses (reviewed by Brown [37]). It was first used to detect rubella IgM in 1981 by Diment and Chantler [38] and Vejtorp [39], who used peroxidase labels, and by Mortimer and colleagues [40] who used a radio-label (125-I). The sensitivity and specificity were later improved by Tedder, Yao and Anderson [41] who used a radio-labelled monoclonal antihaemagglutinin of high affinity to recognize the immobilized antigen, thus increasing the binding ratio and reducing the amount of radioactivity required. Since the necessary instrumentation is now generally available, the capture assay has become the method of choice for rubella IgM detection in the UK. Patients with rashes, pregnant contacts and babies with suspected intrauterine infection can be tested for rubella-specific IgM without restriction.

The original standards for use with the radio-label were prepared by Mortimer and his colleagues on the arbitrary assumption that a pool of sera from recent cases of rubella contained 100 units/ml. One unit then turned out to be a reasonable cut-off level, giving a test ratio (in counts per minute) of about 2·5:1 when compared with negative serum. It would be more rational to adopt the system now in use for certain other IgM capture assays, in which one unit is defined as the proportion of specific IgM that gives a ratio equivalent to three standard deviations of the normal range of activity in negative sera. Serial dilutions of IgM-positive in antibody-negative serum are tested, and the dilution corresponding to one unit is discovered by interpolation. A set of standards (e.g. from 40 to 1 units) is then prepared by making the appropriate mixtures, and these standards, together with the unknown serum, are finally tested at the same single dilution. The unitage of the unknown is discovered by interpolation. At present, laboratories using a radio-label usually replace their expired standards with new mixtures that produce similar results. Laboratories using an enzyme label rely on standards supplied by the manufacturers of kits. The consequent variation in ascribed unitage could be avoided if a national standard serum were available.

Owing to its high sensitivity, the capture assay sometimes gives positive results which do not necessarily indicate recent infection. A few patients produce IgM antibody for months or even years after an acute infection, and this may cause diagnostic difficulty during a subsequent pregnancy if the patient is investigated after contact with rubella – particularly as reinfection itself may stimulate the production of IgM [42]. Some sera which react strongly with rubella antigen in the capture test also react in the corresponding test for IgM antibody to human parvovirus B19, and vice versa [43]. This may indicate the simultaneous presence of IgM antibody to both viruses, but is more often a cross-reaction of unknown mechanism. Cross-reactions can also occur after recent infection with other dissimilar viruses such as EB virus and cytomegalovirus. Misleading results can occur if there is a disturbance of the serum proteins (particularly an increase in globulin) and for purely physical reasons if serum is heat-inactivated before being tested. In many cases no definite cause can be identified. False positive reactions are usually weak and seldom cause serious diagnostic difficulty provided that the IgM results are assessed in conjunction with the clinical data and the results of other serological tests.

Determination of immune status

The determination of immune status is in some ways more difficult than the diagnosis of acute infection because large numbers of sera must be tested with adequate sensitivity and specificity but the minimum of manipulation. For many years the HI test was used, although it was clearly not ideal because all sera had to be diluted and then treated to remove non-specific inhibitors. Kaolin does not always absorb inhibitors completely and the effect of any residue, although unimportant if antibody is plentiful, may be significant if little or no antibody is present. A low result may indicate antibody, non-specific inhibitors, or both, and the consequent difficulty of interpretation makes it advisable to test up to three serial dilutions. What ought to be a simple screen thus becomes a multi-step procedure almost as laborious as a full titration. Antibody and lipoproteins can be physically separated by flotation centrifugation and then titrated independently [44]. A PHLS study in which flotation fractions were stained by IF showed that non-specific inhibitors could exert an effect equivalent to about 12–14 international units of antibody per ml [45]. To allow for this the authors recommended that the minimum immune titre (MIT) by HI should be regarded as at least 24 i.u./ml. This figure, which erred on the safe side, underestimated the prevalence of immunity in serological surveys and probably caused many unnecessary vaccinations.

Screening for immunity became simpler and more accurate with the introduction of radial haemolysis (RH). This was a logical extension of earlier work on gel diffusion, and between 1975 and 1978 methods were reported for detecting antibody to several haemagglutinating viruses. Methods for rubella were described in 1975 by Skaug [46] and Strannegard [47] and their colleagues, and modified in 1980 by a PHLS working party to produce a robust test which could be used routinely on a large scale [48]. Because RH is unaffected by non-specific inhibitors, no absorption is necessary and the MIT can confidently be reduced to 15 i.u./ml. Sera are tested undiluted and the only manipulation consists of inactivation at 60 °C for 20 min. Gels have a shelf life of several days and can be prepared in the laboratory on a weekly basis.

Screening can also be done by latex agglutination (LA) which involves even less manipulation than RH since no inactivation is required. This method is at least as sensitive as RH – perhaps too sensitive, since it will detect less than 15 i.u./ml and the cut-off cannot be adjusted by the user. LA kits with a realistic shelf life are available commercially, and the method is therefore useful for laboratories that do not do enough tests to justify pouring RH plates. For others RH remains the best and cheapest screening test, although LA is a useful backup when RH is difficult to interpret.

Sera can also be screened by ELISA, using either an indirect technique (see above) or a competitive assay. Indirect ELISA requires more manipulation and is unlikely to replace RH for routine screening, although it can be useful in special cases. In the competitive assay wells coated with antigen are treated with undiluted patient's serum to which monoclonal antibody labelled with enzyme is immediately added; any antibody in the patient's serum then competes with the labelled monoclonal and ultimately reduces the production of colour from the substrate. The result depends on the relative titres and affinities of the competing

antibodies, but even if the affinity of the labelled antibody is high, very little patient's antibody is needed to secure virtually 100% inhibition; consequently the dose–response curve covers only a narrow range of low antibody titres and the assay cannot be regarded as quantitative. Since the assay is at its most efficient at the mid-point of the dose–response curve the test should be adjusted so that the chosen MIT gives about 50% inhibition. At present, competitive ELISA appears to be a useful backup rather than a threat to RH.

DIAGNOSIS OF CONGENITAL RUBELLA

Congenital rubella is a chronic infection which remains active for several months after birth until all infected cells have been eliminated. During the first 6 months of life congenital infection is evinced by the presence of specific IgM antibody (since maternal IgM does not cross the placenta) and by the shedding of infectious virus which can be cultured from the throat, urine, cerebrospinal fluid and virtually every organ from which material is available. For many years virus isolation remained the principal method of diagnosis in neonates because IgM could be detected only by fractionation followed by tests for neutralizing [49, 50] or HI [51] antibody. IF tests on sucrose gradient fractions improved the sensitivity but increased rather than diminished the technical complexity [52]. Now, however, the reverse is true and virus isolation, which remains time-consuming and unreliable, has been largely supplanted by improved tests for IgM, using the capture technique with either an enzyme or a radio-label [53].

In children aged between 1 and 4 years the presence of specific IgG antibody has until recently been regarded as evidence of congenital infection on the grounds that IgG derived from the mother has disappeared by the age of 10 months and acquired rubella is uncommon before the age of 4 years. Reports of loss of antibody in congenitally-infected children [54] were almost certainly due to the use of the HI test which detects only anti-haemagglutinin; even when the HI is negative antibody can still be detected by IF [28] – or by ELISA provided that the antigen contains whole virions [55]. However, the presence of antibody at 1–4 years is becoming difficult to interpret in the UK because the extension of the vaccination programme in 1988 to include mass vaccination of boys and girls in the second year of life is already raising the prevalence of vaccine-induced antibody. The augmented vaccination programme should eliminate congenital rubella but until it does so the diagnosis in a dwindling number of congenitally-infected children in this age group will become increasingly difficult.

The availability of sensitive and specific tests for confirming the diagnosis of acquired rubella in pregnant women and congenital infection in babies at risk enabled Miller, Cradock-Watson and Pollock [56] to assess prospectively the separate risks of fetal infection and damage after maternal rubella at all stages of pregnancy. These results are discussed by Miller elsewhere in this journal [57].

CURRENT PROBLEMS

Minimum immune titre

It has been argued that the MIT could be reduced to less than 15 i.u./ml if lower titres could be accurately measured. This proposal was supported by Mortimer and colleagues [58] who found that women with levels of 5–15 i.u./ml did not

produce an IgM response to vaccine and were therefore probably being immunized unnecessarily. The question of retaining or reducing the 15 unit MIT was considered by a recent PHLS working party which failed to reach a unanimous opinion [59]. The arguments centre on the degree of protection which this figure is believed to indicate. Briefly, any circulating antibody indicates some degree of immunity, but neither the firmness of immunity nor the biological quality of the antibody is necessarily proportional to the titre as measured in the laboratory. In general, more than 15 i.u./ml indicates protection, but some proven cases of reinfection leading to congenital rubella have occurred in women with higher titres [60]. Vaccinees, whose titres can sometimes fall to less than 15 i.u./ml [61], are thought to be especially vulnerable. The effect of these variables is still so uncertain that as long as wild virus continues to circulate it seems wiser to retain the MIT of 15 i.u./ml which RH can easily detect. Laboratories using LA should be aware that they may be using a lower cut-off.

Diagnosis of reinfection

Reinfection, usually asymptomatic and confirmed by a rise in antibody titre, can occur after close contact with a case of rubella. Virus is sometimes shed from the throat but viraemia is probably rare. Reinfection during pregnancy is worrying because of scattered reports that it has caused fetal infection. These reports are difficult to evaluate because the secondary character of the mother's infection has usually been inferred from an earlier report of pre-existing HI antibody. Morgan-Capner [62], reviewing this problem in detail, analysed 10 previously published cases: in his opinion 3 vaccinated women who developed intrauterine infection were probably reinfected; 3 of the others, all with rashes, probably had primary rubella and the rest were difficult to interpret. Six further reports [60, 63–67] have added 11 well-documented cases (1 with a rash) leading to fetal infection (3 abortions, 8 badly damaged babies). One of these reports lays down serological and historical criteria for diagnosing reinfection [60]. Since there is now no doubt that reinfection, with or without a rash, can occasionally cause fetal infection, the serological investigation of all pregnant contacts is essential, regardless of their vaccination history, present antibody status or the recorded results of any previous tests. The frequency and outcome of rubella reinfection in pregnancy are at present the subject of a prospective PHLS survey. An earlier study of 34 cases revealed no evidence of fetal infection, which suggests that an adverse outcome is uncommon [68].

The risk of fetal infection is thought to be greatest when a woman with vaccine-induced immunity is reinfected with wild virus. During the next few years mass vaccination of both sexes during the second year of life should eliminate wild virus by increasing the prevalence of immunity in children; in the meantime reinfections will continue to occur and the distinction between primary infection and reinfection is therefore essential. Primary infection is accompanied by IgG seroconversion with a strong IgM response; reinfection is characterized by a rise in the titre of pre-existing IgG antibody with an IgM response which is usually weak but sometimes in the 'primary' range. Discrimination is straightforward if well-timed specimens are available but difficult if specimens have been taken too long after contact. Attempts have therefore been made to distinguish between the different types of IgG antibody formed in these two situations.

The introduction of monoclonal antibodies to IgG subclasses in the 1980s raised hopes of distinguishing between primary infection and reinfection by measuring IgG1 and IgG3 titres separately in an antiglobulin ELISA. Thomas and Morgan-Capner [69] reported that virtually all patients with primary rubella produced both subclasses of antibody. All patients with remote infections had IgG1 and 13% also had IgG3; all patients with reinfections had IgG1 and 31% had IgG3. The subclass profile was therefore not diagnostic in any individual case, although the absence of IgG3 cast doubt on the diagnosis of primary infection.

Early experimental work on antigen-antibody interactions showed that maturation of the humoral immune response was characterized by an increase in antibody affinity. Measurement of avidity in human serum, if it discriminated between 'new' and 'old' antibody, might therefore distinguish between primary infection and reinfection. Equilibrium dialysis, a research method for measuring affinity with hapten antigens, is unsuitable for use with viral antigens and polyclonal human antibodies and is too laborious for diagnostic work. Two simpler methods in current use employ a mild protein denaturant, either to prevent the binding of low avidity antibody or to allow it to be dissociated by washing. In the former method serial dilutions of serum are tested by indirect ELISA in the presence or absence of denaturant; two dose–response curves are drawn and the avidity is assumed to be inversely related to the shift produced by denaturation. Thomas and Morgan-Capner [70], using diethylamine as a denaturant, found that the avidity of IgG1 did not increase after reinfection but was similar to that in sera from past infection. Avidity after primary rubella was always less, provided sera were taken within 28 days of onset. In the second method, which needs only a single dilution of serum, denaturant is added to the washing fluid in order to detach low avidity antibody previously bound to immobilized antigen. The absorbances with and without denaturant are measured, and the former is expressed as a percentage of the latter. Enders [65] and Hedman [71, 72] and their colleagues, using 8 M urea as a denaturant, reported that avidity was low for 4 weeks after the onset of primary rubella but then began to increase; all sera taken more than 10 weeks after the onset were of high avidity and easily distinguishable from those taken in early convalescence. Sera from 17 cases of reinfection were of high avidity – similar to those from patients with remote rather than recent infection.

These changes in avidity are a helpful guide, but as there is still no absolute marker of reinfection the diagnosis in any individual case depends on interpreting current tests in conjunction with the vaccination history and the records of tests performed in the past.

Diagnosis of fetal infection

Since pregnancy is terminated after rubella because of the probability rather than the certainty of fetal damage, prenatal diagnosis of fetal infection, if it could be done early and quickly, would prevent unnecessary abortions. Prenatal diagnosis would be valuable after rubella during the fourth month when the risk to the fetus is falling, and after earlier infection if the diagnosis were in doubt or the parents were reluctant to allow termination; it could also be used in cases where it was impossible to distinguish between primary asymptomatic infection (high risk) and reinfection (low risk). There have been two approaches to this

problem: first, detection of IgM antibody in fetal blood; secondly, examination of chorionic villus biopsies for viable virus, antigen or viral nucleic acid.

Improvements in the techniques of cordocentesis and IgM detection enabled four groups of workers to test fetal blood specimens from more than 60 cases of rubella during the first 4 months of pregnancy [73–76]. IgM was detected at 19–26 weeks in 17 cases and fetal infection was later confirmed in 15 of these by examining abortion material or specimens from the baby at term. Unfortunately, evidence of fetal infection was later found in seven cases in which the fetal IgM test had been negative at 14–22 weeks; consequently a negative result before 23 weeks does not necessarily exclude infection. Even if results became reliable at 23 weeks the delay would be intolerable for the patient and the test would virtually coincide with the legal limit for abortion. This approach is therefore unrealistic.

In 1986 Terry and colleagues [77] isolated virus from a chorionic villus biopsy taken at 12 weeks gestation after rubella in very early pregnancy; they also detected viral antigen (by immunoblotting) and viral RNA (by dot hybridization with a cDNA probe). After termination at 13 weeks they obtained similar results by the same methods from the aborted fetus and placenta. Hybridization, which can be completed in 3–5 days is far quicker than traditional methods of virus isolation; it does not depend on the presence of thermolabile viable virus and it has a target size of only a fraction of the whole genome. A subsequent comparison with virus isolation showed that hybridization detected viral RNA in more aborted fetuses and in more fetal organs after rubella in early pregnancy; virus isolation with a negative hybridization result occurred only in material from the eyes of two fetuses [21]. Hybridization is therefore potentially the better method for examining chorionic villus biopsies with a view to making a quick decision on the management of an individual pregnancy. Hybridization has a sensitivity of about 1–2 pg of viral RNA. For the smallest chorionic villus samples, however, even this level of sensitivity may be insufficient and may need to be increased by the polymerase chain reaction (PCR). Recently Ho-Terry, Terry and Londesborough [76] achieved a gain in sensitivity of about 1000-fold by a combination of target amplification (by PCR) and signal amplification (by Southern blotting). They obtained clear-cut positive results from fetal and placental tissues (including one chorionic villus biopsy) from five out of six patients with first trimester rubella. Further results on chorionic villus samples are awaited with interest.

The possibility of rapid virus isolation should not be completely dismissed. Conventional methods are too slow but viral RNA could perhaps be detected in cell cultures by hybridization or PCR. Viral antigen could also be detected in cell cultures by newer methods. For example Scalia, Gerna and Halonen [78] reported that antigen produced by a laboratory strain of rubella virus could be detected after only 48 h in vero cell culture fluid by time-resolved fluoro-immunoassay. This is a complex technique which is not generally available in the UK but if enhanced luminescence could detect antigen expressed by wild rubella virus then virus isolation might take no longer than hybridization.

FUTURE TRENDS

It is difficult to predict future technical trends because existing methods already provide accurate diagnosis and a reliable base for epidemiological surveys.

Nevertheless, it is a legitimate criticism that current serological tests depend on biological reagents which are vulnerable to non-specific effects and irrelevant antibodies. A cleaner approach would be to identify the epitopes that elicit protective antibodies and then prepare the appropriate peptides by synthesis or bio-engineering. Rubella virus contains three major structural proteins – the capsid protein C and the envelope glycoproteins E1 and E2, of which E1 is the protein mainly involved in reactions with HI and neutralizing antibodies. At least six epitopes have been identified on E1 by monoclonal antibodies. Terry and colleagues [79] have located three distinct epitopes in a short region of 40 amino acids. This clustering suggests that it might be possible to synthesise a standard peptide antigen for diagnostic tests. Suitable antigens might also be prepared by recombinant techniques: so far, the E1 protein has been expressed in *Escherichia coli* [80], E1 and E2 have been produced in baculovirus-infected insect cells [81] and attempts are in hand to express E1 and E2 in mammalian cells (J. M. Best, personal communication). The baculovirus system seems promising, since not only are unwanted antigens less likely to be present but recombinant proteins produced in this way have been found to undergo post-translational changes such as glycosylation and to exhibit many of their normal biological properties [81].

Defined antigens should improve specificity, both in the indirect test for IgG antibody and in the capture test for IgM. ELISA techniques using these antigens could perhaps also be improved by substituting luminescence for the chromogen system: timing in the performance of the test would then be less critical, standardization would be better and doubling dilutions might be unnecessary.

Antibody tests have traditionally been carried out on serum because concentrations in more accessible fluids such as saliva or urine are too low to be detected by conventional techniques. The capture assay, however, being independent of concentration, is well suited for testing a transudate such as saliva which contains, at lower concentrations, the antibodies present in serum. It is particulary good for IgM because the specific component forms, if only temporarily, a large proportion of the total; we can therefore expect to see further developments in salivary IgM tests (especially in children) for the diagnosis of viral infections such as measles, mumps, rubella and other exanthems such as erythema infectiosum (parvovirus B19) with which rubella can be confused. If salivary IgG could also be detected reliably, saliva might replace serum in large-scale antibody prevalence studies [82].

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