the initiating event in acute appendicitis is an infection leading to lymphoid hyperplasia in the appendicular wall and consequent obstruction of the proximal lumen.⁸ Under this hypothesis the epidemic of appendicitis in Britain was a consequence of altered immunity in children, consequent on improved sanitation and hygiene. It is generally agreed that the ultimate event in pathogenesis is invasion of the distal appendicular wall by organisms habitually among the appendicular flora. Diet may influence this by changing the flora. After ingestion of green vegetables an undigested residue reaches the caecum, which may alter the transit time and the substrate of the bacterial flora. Cooked potatoes are digested completely, however, and do not influence the substrate of the caecal flora.¹³ Multiple regression analysis supports the conclusion that the positive correlation with potatoes reflects the inverse relation between their consumption and that of other vegetables which protect against appendicitis.

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First trimester prenatal diagnosis of congenital rubella: a laboratory investigation

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Abstract

Acute primary maternal infection with rubella virus during pregnancy often, but not invariably, leads to the congenital rubella syndrome. Diagnosis by detection of virus specific IgM in the mother is not always possible, and in those cases in which IgM is detected the fetus has not necessarily also been infected. A method for direct, prenatal detection of fetal infection would allow more accurate early diagnosis of congenital rubella syndrome.

In this study a case of suspected preconception rubella infection that was not referred until 14 weeks after the appearance of a rash was studied to determine whether a retrospective serological diagnosis of primary rubella could be made, and whether direct evidence of fetal infection could be obtained from a chorionic villus biopsy specimen by detecting virus specific antigens or ribonucleic acid (RNA) sequences. Monoclonal antibodies and a cloned complementary deoxyribonucleic acid probe were used successfully to detect antigens to rubella virus antigens and RNA sequences in the chorionic villus biopsy specimen, which was taken at 15 weeks' gestation.

This method should serve as a new approach to the diagnosis of congenital rubella syndrome in utero.

Introduction

Primary acute rubella infection in pregnancy often, but not invariably, results in congenital rubella syndrome in the fetus.^{1,2} The diagnosis usually relies on the detection of virus specific IgM in the mother. Serum fractionation followed by haemagglutination inhibition testing usually detects virus specific IgM only up to six weeks after the onset of the rash, though some laboratories report occasional persistence of IgM up to 25 weeks.^{3,4} Consequently, more sensitive immunoassays have been developed for rubella IgM detection,^{5,6} but this has generated two distinct problems. Firstly, the more frequent detection of rubella IgM has inevitably led to an increase in the number of therapeutic abortions being carried out and, therefore, an increase in fetal wastage. Secondly, the low levels of IgM that can now be detected are not necessarily indicative of recent infection and may be present in serum samples obtained from subjects during reinfection as well as months after the acute phase of a primary infection,^{7,8} when the risk to the fetus is minimal.¹

These observations clearly show that diagnosis of congenital rubella by detecting maternal virus specific IgM is not entirely satisfactory and that a method for direct fetal diagnosis is desirable. Such a method should be capable of detecting fetal infection as early in pregnancy as possible for maximum benefit to the patient. Rubella specific IgM can sometimes be shown in fetal blood after maternal rubella infection.^{9,10} This is indicative of fetal infection as maternal IgM does not normally cross the placenta,¹¹ but the usefulness of the method is limited by the late (at 19-22 weeks' gestation) and unreliable development of detectable levels of fetal IgM.¹⁰ Isolation of virus from amniotic fluid¹²⁻¹⁵ is similarly limited as amniocentesis cannot be carried out until at least 16 weeks' gestation, and to this must be added the time required to confirm isolation, which, even if successful, may be several weeks.

We describe the laboratory investigation of a case of suspected preconception rubella infection that was not referred until 14 weeks after the rash had appeared. Our objectives were to determine whether a retrospective serological diagnosis of primary rubella could be made and whether direct evidence of fetal infection could be obtained from a chorionic villus biopsy specimen by detection of

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virus specific antigens or ribonucleic acid (RNA) sequences. If successful this would provide a new method for the diagnosis of congenital rubella syndrome that could give a positive result as early as the ninth week of pregnancy.

Patients and methods

The patient, aged 36, had been seronegative for rubella during her last pregnancy. She remained unvaccinated and two days after her last menstrual period developed a rash that was diagnosed clinically as rubella by her general practitioner. She was referred to King's College Hospital, London, at 11 weeks' gestation. Although this was her fifth pregnancy, she was reluctant to have an abortion as two of her previous pregnancies had ended in intrauterine death.

FIG 1—(a) Analysis of patient's serum by modified IgM capture ELISA technique. Top panel: patient's serum (■—■). Middle panel: IgM negative (○—○) and positive (●—●) control serum samples from pregnant women. Bottom panel: RubEnz MII kit 5 unit positive (●—●) and negative (○—○) serum samples. Bars are ranges ± 2 SD. (b) Detection of rubella specific IgM by ELISA after chromatographic fractionation of patient's serum at 15 weeks' gestation. Top panel: ELISA reading. Bottom panel: Absorbance profile at 280 nm.

VIRUS AND CELLS

Rubella virus, strain "Judith," was grown in baby hamster kidney cells (BHK-21) and purified as previously described.¹⁶ Vero cells were grown in Medium 199, supplemented with 5% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μ g/ml). Haemagglutination inhibition testing was performed as described previously.¹⁷

CLINICAL SPECIMENS

The chorionic villus biopsy specimen¹⁸ was immediately divided into two samples containing fetal tissue only or maternal plus fetal tissue. The samples were checked microscopically and frozen.

All biopsy or aborted tissue specimens were frozen in liquid nitrogen or dry ice as soon as possible (usually within 30 minutes) and stored at -70°C . Fluid specimens were transported at 4°C and processed as soon as possible.

DETECTION OF VIRUS SPECIFIC ANTIBODY

Virus antibody was detected by an indirect enzyme linked immunosorbent assay (ELISA).¹⁹ Enzyme labelled antiglobulins (Sigma) used in this

investigation were preabsorbed with fetal bovine serum, appropriate cell antigens, and rubella virus.

VIRUS ISOLATION

Tissue specimens were teased out in 2 ml of Medium 199, supplemented with 2% fetal bovine serum, 100 IU penicillin/ml, 100 μ g streptomycin/ml, and 25 μ g fungizone/ml and inoculated into four replicate cultures of confluent vero cells. The cells were incubated at 37°C , examined daily for cytopathic effect, and passaged at weekly intervals for a total of three passes. The supernatant from positive cultures was dialysed against coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6), bound to the wells of ELISA trays, and tested for rubella antigens by incubation with rubella specific mouse monoclonal antibodies overnight at 4°C followed by alkaline phosphatase conjugated antimouse IgG raised in sheep (Sigma).

MONOCLONAL ANTIBODIES

A mixture of 11 monoclonal antibodies, recognising three distinct epitopes on the rubella virion, was used to detect rubella specific antigens by immunoblotting. Details of preparation and characterisation of the monoclonal antibodies will be published elsewhere.²⁰

SERUM FRACTIONATION BY FAST PROTEIN LIQUID CHROMATOGRAPHY

Serum was fractionated by ion exchange chromatography on a Mono Q column (Pharmacia).²¹ The collected fractions were diluted 1/10 in phosphate buffer saline-Tween 20 (pH 7.0) and tested for rubella specificity by indirect ELISA.

IMMUNOBLOTTING

Antigens were extracted from clinical specimens in TN (10 mM trometamol-hydrogen chloride pH 7.4, 15 mM sodium chloride) containing 1% Triton X-100 and 0.34% aprotinin (Sigma). The detergent was removed by two extractions with five volumes of ether. Antigens extracted from the same wet weight of test and control clinical specimens were serially diluted and 3 μ l volumes dotted on to a nitrocellulose filter. After drying in air the filter was preincubated in TN containing 3% bovine serum albumin for one hour at 40°C followed by incubation at room temperature for 16 hours with rubella specific monoclonal antibodies in TNEG (50 mM trometamol-hydrogen chloride pH 7.4, 150 mM sodium chloride, 5 mM edetic acid, 0.25% gelatin) containing 0.05% Nonidet P40. All subsequent incubations were at room temperature. After washing in TNEG/Nonidet P40 for 40 minutes the filter was incubated for 16 hours in the same buffer containing 10^6 counts per minute/ml of antimouse globulin labelled with iodine-125 (Amersham International, UK), washed with TNEG containing 0.5% Triton X-100 and 0.1% sodium dodecyl sulphate for four hours, rinsed in water, dried, and autoradiographed at -70°C using X-Omat AR film (Kodak).

Positive control specimens were obtained from aborted products of conception from which rubella virus had been isolated and in which rubella specific antigens and RNA had been previously detected. Negative controls were obtained from a fetus aborted after intrauterine death. Rubella virus could not be isolated from this fetus, and all tests for rubella antigens and RNA yielded negative results.

RNA HYBRIDISATION

RNA was extracted from clinical specimens with 4 M guanidinium isothiocyanate, hot phenol, and chloroform at 60°C .²² RNA derived from the same weight of test and control clinical specimens was dissolved in water and 5 μ l volumes dotted on to a nitrocellulose filter. After drying in air the filter was baked at 80°C for two hours, prehybridised and hybridised²³ using a phosphorus-32 labelled rubella complementary deoxyribonucleic acid (cDNA) probe²⁴ cloned into pAT153 (made under contract to London Biotechnology Limited, University College London). The plasmid, containing a 300 base pair rubella cDNA insert, was labelled to a specific activity of 10^6 disintegrations per minute/ μ g by primer extension.²⁵ Positive and negative control specimens were obtained as above.

Results

RETROSPECTIVE SEROLOGICAL DIAGNOSIS OF MATERNAL RUBELLA

The patient's serum had a haemagglutination inhibition titre of 1/256 at 15 weeks' gestation and contained no detectable heterophilic antibodies.²⁶

Rubella specific IgM was tested in duplicate by IgM capture assay with a RubEnz MII kit (Northumbria Biologicals, UK), following the manufacturer's instructions. The level of IgM detected was 2.8 units, which was equivocal (negative <2.5 units; positive >4.0 units). Even when the pregnancy related haemodilution factor of roughly 80% was taken into account the corrected value of 3.6 units was still in the equivocal range, though it approximated to the level of a low positive. Repeat testing of later serum samples was deemed inappropriate as IgM levels should decline as the acute phase of infection becomes increasingly remote.

FIG 2—Immunoblot of antigens extracted from chorion biopsy specimen and aborted products of conception.

To obtain a more precise result the patient's serum sample and the positive and negative control serum samples were titrated in four replicates, from which the standard deviations could be calculated. The results (fig 1a) show that the patient's serum did contain a low level of rubella specific IgM.

Further confirmation of the presence of rubella specific IgM was obtained when the patient's serum sample was fractionated by chromatography and eluted fractions containing IgM but not IgG²¹ were shown to be positive by ELISA (fig 1b). This procedure ruled out the possibility of a false positive result due to the presence of rheumatoid factor and allowed quantitation of the patient's total serum IgM concentration.

PRENATAL EVIDENCE FOR FETAL INFECTION

Having established the laboratory diagnosis of maternal rubella infection, we analysed a chorionic biopsy specimen, taken at 12 weeks' gestation, for the presence of rubella antigens and RNA. The immunoblot of the antigens extracted from the chorionic villus sample gave a strong signal with the rubella specific monoclonal antibody pool (fig 2, lanes 11 and 12). The sample that contained only fetal tissue gave a stronger signal than the sample

containing both fetal and maternal tissue, suggesting that virus was replicating preferentially in the fetal tissue.

Using a cloned rubella cDNA probe to detect rubella specific RNA sequences by dot hybridisation, we obtained a similar result, indicating more extensive viral RNA synthesis in the fetal tissue (fig 3a, lanes 1 and 2).

INVESTIGATIONS AFTER ABORTION

After vaginal termination of pregnancy at 13 weeks' gestation we examined the products of conception using the same techniques that had provided prenatal indications of fetal infection, and, in addition, we tried to isolate rubella virus from the aborted fetus and placenta.

The patient's serum haemagglutination inhibition titre at the time of termination had dropped to 1/128 (see table), and virus specific IgM was no

TABLE 1—Results obtained from specimens obtained before and after abortion

Test	Prenatal specimen	Specimen after abortion
Serology:		
Haemagglutination inhibition titre	1/256	1/128
Fast protein liquid chromatography and ELISA	Positive IgM	Negative IgM
Virus detection*:		
Isolation in vero cells	Not done	Placenta, fetus
Immunoblot	Chorion	Placenta, amniotic fluid, fetus
Hybridisation	Chorion	Placenta, amniotic fluid, fetus

*Results expressed as isolates that yielded positive results.

longer detectable. Immunoblotting and hybridisation provided strong confirmatory evidence of virus infection in the aborted placenta (fig 2, lane 8; fig 3b, lane 2), amniotic fluid (fig 2, lane 6; fig 3b, lane 4), and fetus (fig 2, lane 10; fig 3b, lane 3). In addition, rubella virus was isolated from both tissue specimens (see table). We did not attempt to isolate virus from the amniotic fluid. These results confirmed the diagnosis of fetal rubella

FIG 3—Hybridisation of cloned rubella cDNA probe to RNA extracted from patient's chorion biopsy specimen and aborted products of conception.

infection, which had initially been based on the chorionic villus biopsy examination. The table gives a comparison of the results obtained from the samples taken before and after termination of pregnancy.

Discussion

The vaccination programme carried out in the United Kingdom has not yet eliminated congenital rubella, and therapeutic abortion on the grounds of detection of maternal rubella specific IgM remains the most practical, though not always the most desirable, means of control. Detection of virus specific IgM is not always possible, however, if the time since infection is too great.

Moreover, detection of maternal rubella specific IgM cannot always solve the problems encountered in the diagnosis of congenital rubella. In this case the time of conception could only be imprecisely dated as the first day of the patient's last menstrual period. Thus the time of conception in relation to the acute episode of rubella infection was not clear, and the probability of fetal infection was difficult to assess. In addition, the patient was reluctant to have her pregnancy terminated. A direct measure of fetal infection was desirable, and in this patient a chorionic villus biopsy examination provided the confirmation that was sought. The size of the biopsy specimen obtained was small; evidence of rubella infection was possible only with the sensitive and highly specific immunoblot and hybridisation tests for rubella virus recently developed in our laboratory. These tests can provide results within three days. Although at this stage the techniques may not be suitable for use in routine diagnostic laboratories because of the requirement for ^{125}I and ^{32}P radioactivity, these difficulties will be overcome when non-radioactive labels of comparable sensitivity become available.

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SHORT REPORTS

Generalised allergic reaction to human insulin

I report a case of local and generalised allergy to Human Monotard insulin that did not recur with transfer to highly purified bovine insulin.

Case report

A 71 year old woman had been an insulin dependent diabetic for 50 years without any history of allergy. Since 1971 her diabetes had been well controlled by neutral insulin (Neusulin) and semilente/lente insulin (Neulente). In September 1983 an infection in her left foot led to amputation of the fifth toe. Her diabetes was well controlled throughout this episode. She was readmitted to hospital in January 1984, having hit her left foot during a fall caused by hypoglycaemia; by 8 February gangrene was apparent in the fourth toe. Her regimen of highly purified Neusulin and Neulente was changed to a sliding scale of Human Actrapid, of which she required 40 units a day. The gangrene deteriorated and on 23 February her left leg was amputated below the knee. After surgery her insulin requirements decreased, and by 13 March she was better and her wound had healed well; she was receiving 12 units of Human Actrapid three times daily. On 19 March she started taking Human Monotard 14 units in the morning and Human Actrapid 10 units twice daily. Two days later she developed a severe eczematous urticarial rash with itching on both upper arms, both legs, the chest, neck, and face. She was mildly feverish (temperature 37.2°C) but did not have associated dyspnoea or wheezing. Treatment was continued, and the next day the rash increased in size, becoming more confluent and remaining intensely pruritic. Its allergic nature was confirmed by a consultant dermatologist,

and the Human Monotard was stopped but the Human Actrapid continued. Over the next 10 days the rash resolved and she was transferred to her original regimen of Neusulin and Neulente before being discharged.

In prick tests performed after her discharge she did not respond to the following insulins (one unit in 1 ml of physiological saline): Neulente, Semitard, Ultratard, human isophane, Human Protaphane, Semitard MC, protamine zinc, and Human Actrapid. She did not respond either to zinc acetate or to the diluents used for Human Actrapid and Human Monotard, but doses of 0.1 ml of one unit of Human Monotard in 1 ml of physiological saline produced large wheal and flare reactions visible for several hours afterwards.

Investigations—A full blood count showed: haemoglobin 129 g/l, leucocyte count $11.3 \times 10^9/\text{l}$ (85% neutrophils, 10% lymphocytes, 4% eosinophils), erythrocyte sedimentation rate 23 mm in the first hour, platelets $325 \times 10^9/\text{l}$. On readmission insulin specific IgE was below the limit of detection (0.2 U/ml) and insulin specific IgG concentration was 0.204 mU/ml.

Comment

Generalised and local reactions to purified and human insulin have been observed^{1,2}; these usually occur after previous exposure to conventional insulins, though cases without previous exposure have been described.³ Wheal and flare reactions to testing are also recognised as a dermal hypersensitivity reaction to insulin.⁴ Patients with generalised insulin allergy usually have high concentrations of insulin specific IgE, but this does not seem to occur with local reactions to insulin.⁵ The mechanism of this patient's reaction remains unclear because it did not occur with Human Actrapid or highly purified bovine insulin and therefore cannot have been a straightforward allergy to insulin. It seems that human insulins, though