**In vitro** propagation of parvovirus B19 in primary foetal liver culture

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The culture of parvovirus B19 in foetal liver tissue has been described recently. We have established the technique in our laboratory and studied parameters affecting the yield of B19 virus. Replication of the virus was detected by radioimmunoassay for B19 antigen and dot blot hybridization assay of B19 DNA, and the virus was localized by immunofluorescence and thin section electron microscopy. B19 DNA and antigen production became detectable at day 2 and reached a maximum at day 5. Virus particles were seen mainly in cell nuclei, but some cytoplasmic membranes were lined with virus particles. The amount of virus produced depended on the age of the foetus and the cell culture and the concentration of erythropoietin and interleukin 3 in the culture medium.

Parvovirus B19, first identified by Cossart et al. (1975), is associated with a wide range of clinical symptoms. It is the cause of erythema infectiosum (Anderson et al., 1983), of transient aplastic crises in patients with underlying haemolytic disease (Pattison et al., 1981) and of chronic bone marrow infection in immunocompromised patients (Kurtzman et al., 1987). Parvovirus B19 infection in pregnancy may lead to hydrops foetalis and foetal loss (Brown, 1989).

Parvovirus B19 is difficult to grow in the laboratory. A range of primary cell cultures and continuous cell lines have been tried, but with very limited success (Young et al., 1984). It is known, however, that the main target cells are erythroid progenitors (Mortimer et al., 1983). The use of foetal livers as a source of haemopoietic cells for reconstitution of bone marrow was suggested many years ago (Russell et al., 1956) and recently Yaegashi et al. (1989) reported the use of foetal liver as a source of erythroid progenitors for the propagation of B19. The studies described in this paper confirm their results.

Liver tissues from suction-aborted human foetuses (gestational range 11 to 17 weeks) were washed in RPMI 1640 (Gibco) and finely minced, before being dispersed in 1 mg/ml collagenase type IV (Sigma). Hepatocytes and non-dissociated clumps of cells were removed by centrifugation. The mononuclear cells, collected from the interface after centrifugation over Ficoll-Paque (Pharmacia), were washed three times and resuspended in Iscove’s modified Dulbecco’s medium (IMDM, Flow Laboratories) supplemented with 20% foetal calf serum, 1 unit (U)/ml of recombinant human erythropoietin (EPO, Epoietin alfa, a gift from Cilag) and 10 U/ml of recombinant interleukin 3 (IL3, British Biotechnology). After overnight incubation at 37 °C, non-adherent cells were removed and aliquots of cell suspension (between 10^5 and 10^6 cells/ml) were inoculated with B19 antigen-positive serum plus additional IL3 (100 U/ml) to stimulate cell division.

A growth curve was obtained by inoculating a 10 ml cell suspension with B19-positive serum (final dilution 10^{-3}), distributing it into 200 μl volumes, and collecting four identical samples at daily intervals for 10 days. Aliquots (20 μl) of supernatants and cell extracts (disrupted by sonication) were tested for B19 antigen by radioimmunoassay (RIA) as previously described (Cohen et al., 1983). Antigen was detected using two B19 mouse monoclonal antibodies (MAbs), VRL/B19/7 and VRL/B19/11, followed by 125I-labelled anti-mouse IgG. Results were expressed as the ratio of counts given by test sample/counts given by uninoculated cell suspension (T/N). The cut-off value was a T/N of 2-0. B19 antigen production was detected by both MAbs, but VRL/B19/7 was more sensitive than VRL/B19/11 (Fig. 1). An increase in antigen reactivity was seen by day 2 and peaked on day 6.

Production of B19 DNA followed a similar time course. In order to detect newly synthesized B19 DNA, the virus was inoculated into cell cultures and allowed to bind for 1 h at 4 °C before the cells were washed twice and resuspended in fresh culture medium. Supernatant fluid was taken at intervals over the next 12 days and examined for production of B19 DNA by dot blot hybridization (Mori et al., 1989) with a B19-specific DNA probe labelled with digoxigenin (Boehringer Mannheim). The amount of B19 DNA was estimated by reference to a standard curve derived by testing dilutions...
of a control serum containing a known quantity of B19 virus. Viral DNA was not detectable on day 0, but was detectable on day 2. By day 12 over 1 μg/ml of B19 DNA was detected in the supernatant, a more than 1000-fold increase.

Production of B19 virus was confirmed by examining the infected cells for B19 antigen by immunofluorescence (IF) using either anti-B19 polyclonal serum or mouse anti-B19-specific MAb (VRL/B19/7; Cohen et al., 1983). IF was not detectable for at least 24 h after inoculation but became visible by 48 h. It was initially confined to the nucleus, but became increasingly cytoplasmic. The proportion of positive cells reached a maximum by day 6, but varied with each culture. The percentage of positive cells varied with the gestational age of the foetus and the period of time between cell preparation and inoculation of the culture. Cell cultures obtained from foetuses aborted at earlier stages of gestation tended to give a higher proportion of positive cells by IF as well as higher yields of virus antigen by RIA. In a liver preparation from a foetus with a gestational age of 10-6 weeks, 10% of the cells were infected with B19 (as determined by IF), compared to less than 1% of cells in foetuses aged 17 weeks or more. Cell cultures inoculated the day following cell preparation produced higher yields of B19 antigen than cells inoculated later (data not shown).

We used livers from foetuses aborted at an earlier stage of gestation than Yaegashi et al. (1989) (11 to 17 weeks compared with 18 to 21 weeks) and this may account for the higher percentage of cells it was possible to infect with B19 in our study (up to 10% compared to 5%).

Weeks 8 to 16 are the period of maximum increase in the haemopoietic potential of the foetal liver (Wintrobe et al., 1981).

Cells from a B19-infected culture were also examined by electron microscopy of thin (40 to 50 nm) sections stained with uranyl acetate and lead citrate. Magnifications were calibrated using catalase. At 4 days the B19-infected cells exhibited margination of chromatin and extreme degeneration of the cytoplasm as the main c.p.e. (Fig. 2a and c). Approximately 1% of the cells contained virus particles which were mostly located in the nucleus (Fig. 2a and b). Virus particles were also seen lining cytoplasmic membranes (Fig. 2c and d). Most cytoplasmic virus particles and many intranuclear particles appeared as 22 nm diameter rings with an electron-lucent centre and were apparently empty. Full, electron-dense, 22 nm virus particles were seen occasionally in the cytoplasm and more frequently in nuclei. An unoinoculated control culture contained no cells showing c.p.e. and no virus particles were seen.

Foetal liver cultures were also used to examine the factors affecting the yield of virus. Cells were prepared and after overnight culture with 1 U/ml of EPO and 10 U/ml of IL3 were washed and resuspended in IMDM with various concentrations of EPO and IL3. B19 virus was added and the cells were incubated for 5 days before cytocentrifugation and examination by IF (Fig. 3a). Virtually no cells were positive by IF in the absence of EPO (one culture only had five positive cells) and few cells (<1%) were positive in the absence of IL3. The percentage of positive cells was maximal with 1 to 5 U/ml of EPO and 100 U/ml of IL3. Higher levels of IL3 or EPO appeared to decrease the number of positive cells. Supernatants from the cultures were tested for B19 antigen by RIA (Fig. 3b). The T/N ratios in the absence of EPO were significantly different (P < 0.001) from those with any amount of EPO. The differences between the T/N ratios obtained for either different concentrations of EPO, or the presence or absence of IL3, were not statistically significant.

All attempts to culture B19 have shown the absolute requirement for EPO and our present study confirms this. The role of EPO in B19 propagation has not, however, been elucidated. It may be important in maintaining cells at the susceptible stage of differentiation, or it may have a more direct role in B19 replication. The presence or absence of IL3 was not as critical, although at optimal levels of IL3 (100 U/ml) the number of inoculated cells which were B19-positive by IF increased, as did the amount of B19 antigen released (the latter was not statistically significant). Takahashi et al. (1990) showed similar findings with bone marrow culture of B19 virus but they used IL3 in the presence of only suboptimal levels of EPO (0.03 U/ml). In this study...
Fig 2. Thin section electron microscopy. (a and c) Infected cells showing degenerate cytoplasm and nuclei with margined chromatin (bar markers represent 500 nm). (b) Higher magnification of the area indicated in (a) showing intranuclear virus particles (bar marker represents 100 nm). (d) Higher magnification of area indicated in (c) showing virus particles aligned on cytoplasmic membranes (bar marker represents 100 nm).
Fig. 3. Yield of B19 antigen from cultures of 200 μl of cell suspension in media containing various concentrations of EPO and IL3. The suspensions were inoculated with B19 (final dilution 10⁻³), incubated for 5 days and examined by IF and RIA. (a) Proportion of positive cells by IF, (b) T/N ratio obtained by RIA for B19 antigen. Concentration of EPO: □, 0 U; ■, 0.3 U; ●, 1 U; ▪, 5 U; □, 10 U.

the amount of B19 DNA released into the supernatant was approximately 50 times that described by Yaegashi et al. (1989) (> 1 μg/ml compared to 20 ng/ml). This may reflect our use of less mature livers for production of the primary cultures. The amount of B19 DNA released was more than 100 times that from bone marrow culture (Ozawa et al., 1987).

Despite the higher yield of virus compared to earlier attempts at B19 culture, it is not envisaged that this method will suffice as a source of diagnostic antigen; foetal tissues are not readily available, the cultures have only short viability and the technique is labour-intensive. Techniques involving transfection of B19 DNA into eukaryotic cells e.g. Chinese hamster ovary cells (Kaji-gaya et al., 1989) or COS-7 cells (Beard et al., 1989), the production of recombinant antigen in Escherichia coli (Sisk & Berman, 1987) and in baculovirus (Brown et al., 1990), and the use of synthetic B19 peptides (Fridell et al., 1989) are all expected to provide much larger amounts of antigen for diagnostic assays.

Methods for culturing B19 in the laboratory will still be required however, both to increase our understanding of the pathogenesis of B19 infection and to allow studies of virus infectivity. Symptomatic B19 infection has been associated with exposure to blood products (Lyon et al., 1989) but until now there has been no method for monitoring viral inactivation in them. Similarly, the role if any of B19 neutralizing antibodies, particularly in patients at risk of developing persistent infection, cannot be assessed without an in vitro culture method. Foetal liver culture allows progress in these areas. Its value is limited because it only permits short term primary cell culture, but a stable cell line permissive for B19 infection may soon be feasible.

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References


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