Replication and Expression of Rubella Virus in Human Lymphocyte Populations

By Janet K. Chantler and Aubrey J. Tingle

Division of Medical Microbiology and Department of Paediatrics, University of British Columbia, Vancouver, B.C., Canada

(Accepted 15 May 1980)

Summary

Human mononuclear cells (lymphocytes and monocytes) from peripheral blood were examined for their ability to support the replication of rubella virus (RV) after infection in vitro. Replication was shown to occur in mixed lymphocytes and to be enhanced by stimulation with phytohaemagglutinin or pokeweed mitogen. A comparison of RV polypeptide synthesis in lymphocytes and RK13 cells showed no major differences in the polypeptides induced by infection. However, cellular translation was inhibited in the lymphocytes facilitating identification of virus polypeptides and eliminating the need for hypertonic labelling conditions used with RK13 cells. RV replication was also shown to occur in sub-populations of T-cells but not in B-cells. However, RV could be rescued from the B and monocyte population by co-cultivation with RK13 cells.

Introduction

Human lymphocytes from peripheral blood (PBL) have been shown to support the replication of a number of viruses in vitro, including mumps virus (Duc Nguyen & Henle, 1966), measles virus (Joseph et al., 1975), vesicular stomatitis virus (Edelman & Wheelock, 1968) and herpes simplex virus (Bouroncle et al., 1970) if stimulated with a mitogen such as phytohaemagglutinin (PHA). In addition, virus isolation from lymphocyte populations has been well-documented in many acute natural infections, and also during persistent infection with measles (Horta-Barbosa et al., 1971), cytomegalovirus (Lang & Noren, 1968) and others. Thus, it is likely that lymphocytes form one of the major sites of virus replication and persistence in humans; they are readily available to a virus during the viraemic phase and the depression of lymphocyte responsiveness to antigen or mitogen which accompanies infection may provide the virus with a mechanism of escape from immune surveillance.

In the case of rubella virus, replication in human PBL in vitro has not previously been shown to occur, although the virus has been isolated from lymphoid tissue after acute natural infection (McCarthy et al., 1963; Heggie & Robbins, 1964) or vaccination (Buimovici-Klein & Cooper, 1979), and also during persistent infection in cases of congenital rubella (Simons & Jack, 1968). In addition, a number of studies have provided indirect evidence of RV replication in human PBL in vitro by documenting the ability of infectious but not u.v.-inactivated virus to depress the proliferative response of lymphocytes to antigens or mitogens as measured by 3H-thymidine incorporation (Montgomery et al., 1967; Maller & Soren, 1977).
This study was therefore undertaken to confirm the long-suspected ability of mixed populations of human lymphocytes to support RV replication and to identify the cell-type(s) within this population in which replication occurs. In addition, the degree of expression of RV polypeptides was examined and compared with that previously reported to occur in RK13 cells (Chantler, 1979).

METHODS

Cells and virus. A plaque-purified wild-type strain of RV ("Thomas") and a mycoplasma-free line of RK13 cells were obtained from Professor B. P. Marmion, University of Adelaide, Australia. Stocks of virus were prepared in RK13 cells infected at input multiplicities of > 1 infectious unit/cell and incubated in M199 + 5% inactivated foetal calf serum (FCS). The supernatant was harvested on days 3 and 5 post-infection and centrifuged at 3500 rev/min in a Sorvall GSA rotor to remove gross cell debris before storage at -70°C. Virus yield was determined by microfocus development in RK13 cells (Kouri et al. 1974) and was usually 10^6 to 10^7 microfocus-producing units (m.p.u.)/ml.

Peripheral blood lymphocyte cultures. Mononuclear cells were obtained from heparinized venous blood samples of normal adults (having rubella haemagglutination inhibition titres of 1:16 to 1:64) by Ficoll-hypaque gradient centrifugation. They were washed in standard medium and cultured in RPMI 1640 supplemented with 15% inactivated FCS and 20μg/ml gentamicin and containing 50μg/ml phytohaemagglutinin (PHA) or 50μg/ml pokeweed mitogen (PWM).

Preparation of lymphocytes from tonsils. Cells were teased from fresh human tonsillar tissue into 5 ml RPMI growth medium, 10% foetal calf serum containing penicillin, streptomycin and mycostatin. The cell suspensions were diluted 1:20 with growth medium and passed through a 60 mesh stainless-steel screen. Crude lymphocytes were then obtained by centrifugation on Ficoll-hypaque.

Purification of T+B lymphocytes

T+B cells were prepared from mixed lymphocyte samples obtained either from peripheral blood or human tonsillar tissue by Ficoll-hypaque gradient centrifugation. The T+B sub-populations were purified by E-rosette formation with sheep red blood cells (SRBC), essentially as described by Keightley et al. (1976).

T cells: 10 × 10^6 lymphocytes (from peripheral blood) in MEM + 20% heat-inactivated foetal calf serum (HIFCS) were mixed with SRBC in a ratio of 2% SRBC to 1 × 10^6 lymphocytes. They were immediately centrifuged at 50 g for 10 min and then incubated at room temperature for 2 h. The mixture was layered on a Ficoll-hypaque gradient and after centrifugation at 400 g for 40 min, the pelleted E-rosette fraction was collected. This was resuspended in ice-cold ammonium chloride buffer (NH_4Cl 0.8%, KHCO_3 0.1%) for 1 to 2 min to allow red cell lysis to occur, then an equal volume of growth medium (MEM + 10% HIFCS) was added and the T cells were centrifuged, washed and resuspended in growth medium.

B cells: 2 × 10^7 mixed lymphocytes from peripheral blood or human tonsils were mixed with 4% SRBC in RPMI + 20% HIFCS and centrifuged at 50 g for 10 min. The mixture was incubated at 4°C overnight and then the B population cells were obtained by density centrifugation on Ficoll-hypaque. The cells at the interface were washed and resuspended in growth medium, and in some cases were treated with carbonyl-iron to remove monocytes.

Characterization of lymphocyte populations. Characterization of cell-types in the sub-populations was carried out as described by Hoffman & Kunkel (1976). T cells were identified by rosette formation with SRBC and B cells by detecting surface Ig receptors (SIg) by direct
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Immunofluorescence. The FITC-conjugated goat anti-human Ig for this test was obtained from Cappel Laboratories, Cochranville, Pa., U.S.A. The presence of monocytes was determined using a non-specific esterase stain (Koski et al. 1976).

Infection and labelling of lymphocytes. Lymphocytes were cultured for 72 h in the presence of PHA or PWM prior to RV infection. In most experiments, $5 \times 10^6$ cells were infected with 5 m.p.u./cell RV in RPMI 1640 + 2% inactivated FCS. After a 2 h adsorption period, the cells were pelleted and the medium changed to RPMI 1640 + 5% inactivated FCS supplemented with the appropriate mitogen. The cells were labelled with 5 to 10 $\mu$Ci $^{35}$S-methionine per culture in 2-5 ml MEM (1/5 Met) medium. At harvest, they were washed once in cold PBS prior to storage at $-20^\circ$C for gel analysis.

Co-cultivation with RK13 cells. B population cells (B lymphocytes and monocytes) were isolated after 3 days mitogenic stimulation in mixed culture and were mock-infected or infected at 5 m.p.u./cell. At 72 h p.i. they were co-cultivated with RK13 cells in Petri dishes or on glass coverslips. The coverslips were harvested on day 5 post-co-cultivation and fixed in acetone for fluorescence studies, while the Petri dishes were labelled with $^{35}$S-methionine for 6 h on day 5 post-co-cultivation before poly peptide analysis. The media of all cultures were also stored for infectious virus assay. Mock-infected or infected mixed lymphocytes (ML) were co-cultivated in an identical manner, but in this case the cells were labelled and coverslips harvested on day 1 and day 3 post-co-cultivation instead of day 5.

Polyacrylamide gel analysis. A modified version of the Laemmli discontinuous buffer system was used (Laemmli, 1970). Before electrophoresis, proteins were denatured in 0.05 M-tris-HCl, pH 7.0, 2% SDS, 5% 2-mercaptoethanol, 0.005% bromophenol blue and 10% glycerol. All samples were heated at 100$^\circ$C for 1 to 2 min and were electrophoresed on a 9.5% polyacrylamide gel slab [0.375 M-tris-HCl, pH 8.8, 0.1% SDS, 0.03% (v/v) TEMED, 0.03% (w/v) ammonium persulphate, 9.5% acrylamide, 0.27% methylene-bis-acrylamide] for 5 h at 25 mA/gel. Following electrophoresis, the gels were dried on to filter paper (Whatman no. 3) and then placed in contact with a sheet of Kodak KP/XR-1 X-ray film in a light-tight folder under pressure for an exposure time of approx. 10 days. The films were developed in a Kodak RP X-Omat processor.

RESULTS

Replication of RV in human lymphocytes

The ability of peripheral blood lymphocytes (PBL) from a normal adult to support replication of RV following stimulation with phytohaemagglutinin (PHA) or pokeweed mitogen (PWM) for 48 to 72 h in culture was examined. RV, released into the medium, was assayed by the production of microfoci in RK13 monolayers as described elsewhere (Kouri et al. 1974) on sequential days after inoculation of $5 \times 10^6$ lymphocytes with 1 to 5 m.p.u./cell (Fig. 1).

RV replication could be demonstrated in lymphocytes after stimulation with either PHA or PWM, although higher titres were found in the cultures treated with PHA. Unstimulated lymphocytes also appeared to support a low level of replication, possibly due to spontaneous transformation in culture or to mitogenic stimulation by foetal calf serum or membrane fragments in the virus inoculum. In parallel experiments, in which lymphocytes were infected immediately after cultivation in vitro and subsequent treatment with PHA, very little, if any, replication of RV occurred. This repression of virus replication in fresh lymphocyte cultures has been reported in several systems including poliovirus (Gresser & Chany, 1964) and mumps virus (Duc-Nguyen & Henle, 1966). It has been variously explained as being an 'interferon effect' or due to a requirement for ongoing cellular RNA or DNA synthesis (Wheelock & Toy, 1973).
Expression of RV-induced polypeptides in PBL

The identification of polypeptides induced by RV during acute infection of RK13 cells has recently been reported (Chantler, 1979). At least 10 virus-specific polypeptides ranging in mol. wt. from 109 to 24.5 K have been identified using a 'hypertonic initiation block' to reduce the background of cellular synthesis. The migration pattern of these on 9.5% polyacrylamide gels is shown in Fig. 2(a to e) to facilitate comparison of RV expression in RK13 with that found in peripheral blood lymphocytes (Fig. 2f, g). In both cases labelling was from 72 to 78 h p.i., but while the RK13 cells were incubated under both isotonic and hypertonic conditions, high-salt was not used in the lymphocyte cultures. A comparison of (e) and (g) in Fig. 2 shows quite clearly the gross similarity in RV polypeptides induced in the two cell types. Moreover, it is apparent that RV inhibits cellular protein synthesis in human lymphocytes to a degree comparable with that obtained by high-salt labelling of RK13 cells (d and e). This inhibition of host translation is in agreement with the known ability of RV to reduce the mitogenic response of lymphocytes infected in vitro or in vivo (Maller & Soren, 1977). It has greatly facilitated the identification of RV polypeptides in lymphocytes where high-salt cannot be used as initiation of message translation appears to be completely inhibited, even in 100 mM excess NaCl.

The RV polypeptides which can readily be detected in PBL cultures include: p75, p50, p46.5, p40 and p32.5. In addition to p75, a second polypeptide of mol. wt. approx. 78 K is frequently detected in lymphocyte cultures, which may represent an extra intermediate in the cleavage process which occurs post-translation. RV polypeptides are believed to be synthesized from a polycistronic mRNA and to undergo a process of post-translational cleavages dependent on host proteolytic enzymes. Any slight difference in the characteristics
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Fig. 2. RV expression in PBL or RK13 cells. A comparison between polypeptides synthesized in mock-infected (M) or RV-infected (I) RK13 cells and PBL labelled 72 to 78 h p.i. is shown. The RK13 cultures were incubated under isotonic (a, b) and hypertonic (c, d) conditions (150 mM-excess NaCl) and RV-induced polypeptides are labelled according to their mol. wt. In a second autoradiogram, gel analysis of polypeptides synthesized in PBL is shown (f, g) and compared directly with the pattern in hypertonically labelled RK13 cells (e).

of the enzyme involved can thus affect the nature or half-life of the cleavage products. A second difference in RV polypeptides can be seen in the species of mol. wt. in the range 46 to 48 K. In RK13 cells, three polypeptides can be detected, p46, p46·5 and p48, of which p46 is the major species (Fig. 2e). In lymphocytes, however, the major species present is p46·5 while the other two polypeptides are present in very small amounts. This may be a cleavage effect or represent a difference in some other post-translational modification such as glycosylation or phosphorylation.
RV polypeptide synthesis early in infection of PBL

The polypeptides synthesized before 24 h p.i. in RV-infected lymphocytes were examined to see whether any early RV polypeptides could be detected. Although RV translation is polycistronic it is possible that, as in the case of the α-viruses, the incoming virion RNA is only partially translated from a terminal initiation site to yield one or several early non-structural proteins; the major late proteins may then be dependent on the synthesis of a 26S positive-strand RNA species which is known to accumulate late after infection in cells infected with both the α-viruses (Simmons & Strauss, 1974) and RV (Sedwick & Sokol, 1970).
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Table 1. Titre of infectious virus in the supernatant of mixed lymphocyte cultures and isolated T + B cells at 72 h p.i.

<table>
<thead>
<tr>
<th>E-rosetting</th>
<th>% SIg*</th>
<th>Monocytes†</th>
<th>Infectious virus titre (m.p.u./ml) 72 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unseparated</td>
<td>51</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>T population</td>
<td>75</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>B population</td>
<td>1</td>
<td>40</td>
<td>53</td>
</tr>
<tr>
<td>B population (carbonyl-iron treated)</td>
<td>1</td>
<td>70</td>
<td>1</td>
</tr>
</tbody>
</table>

* SIg - surface immunoglobulin.
† Monocytes - as determined by non-specific esterase staining.

 Cultures were infected at high multiplicity (about 10 m.p.u./cell) and labelled from 0 to 6 h, 0 to 16 h and 0 to 24 h before polypeptide analysis. No RV polypeptides were detected in the 0 to 6 h and 0 to 16 h pulses and in Fig. 3 only the 0 to 24 h sample is shown, together with the profiles from mock-infected cells and cells labelled 48 to 72 h p.i. No specifically early proteins were detected; however, a small amount of several late species can be distinguished (p75, p50, p46·5 and p32·5), indicating that structural protein synthesis commences between 16 and 24 h p.i. The absence of detectable amounts of any early species does not rule out the possibility that low levels of one or several polypeptides are synthesized, but their identity will require the isolation of ts mutants of RV, or analysis of the products of an in vitro translation system.

RV replication in T and B lymphocyte sub-populations

Sub-populations of peripheral blood lymphocytes were prepared by rosette formation with sheep red blood cells (SRBC), basically as described by Keightley et al. (1976). The degree of purification of each sub-population was assessed by determining the percentage of rosetting cells or the percentage possessing surface immunoglobulin (SIg) markers in each. Monocytes were also assayed by non-specific esterase staining of cells in each population. The results are shown in Table 1.

The isolated T and B populations (before or after carbonyl-iron treatment to deplete monocytes) were treated for 3 days with PHA or PWM respectively, and were then infected with RV at 1 to 5 m.p.u./cell. The amount of supernatant virus was assayed at 72 h p.i. and the cells were labelled with ³⁵S-methionine from 72 to 78 h p.i. to investigate the synthesis of RV polypeptides. The results of microfocus assay (Table 1) indicate that replication of RV occurred in T cells resulting in titres of supernatant virus comparable to those found in mixed lymphocytes (ML). However, no replication was demonstrated in the B cell population (with or without carbonyl-iron treatment) which showed titres of supernatant virus of < 10⁸ m.p.u./ml. Analysis of RV polypeptide expression in T cells and monocyte-depleted B cells, shown in Fig. 4, further substantiated these findings. The major RV polypeptides identified in ML were also found in T cells (Fig. 4b) but not in B cells (Fig. 4d). Not even partial expression of RV was found in the B lymphocytes, as might have been expected if there had been a block in replication during RNA synthesis or during the cleavage process.

In view of the poor mitogenic stimulation of B cells by PWM which occurs in isolated populations (Gmelig-Meyling et al. 1977; Insel & Merler, 1977) and which might be responsible for the lack of RV replication, two modifications of procedure were tested in an attempt to improve B cell stimulation. First, the mixed population was incubated in the
Fig. 4. RV expression in T and B cells. Analysis of polypeptides synthesized in sub-populations of T and B cells, mock-infected (M) or infected (I) with RV. Virus polypeptides in T cells are indicated, with their mol. wt. (b). None is detectable in the B cell population (d).

presence of mitogens (PHA or PWM) for 3 days before separation of the sub-populations and immediate infection. Secondly, the isolated B cells were incubated in Marbrook chambers in order to allow co-operation with mixed lymphocytes in the inner chamber. While both these procedures improved the ability of isolated B cells to incorporate $^{35}$S-methionine, no evidence was found for the synthesis of any virus-induced polypeptides or infectious virus.
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Table 2. Detection of cytoplasmic fluorescence and infectious virus in cultures of B cells or mixed lymphocytes (ML) co-cultivated with RK13 cells

<table>
<thead>
<tr>
<th></th>
<th>Infectious virus (m.p.u./ml)</th>
<th>Cytoplasmic fluorescence</th>
</tr>
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<tbody>
<tr>
<td>ML/RK13 co-cultivation</td>
<td>$1.9 \times 10^6$</td>
<td>+</td>
</tr>
<tr>
<td>B cell/RK13 co-cultivation</td>
<td>$5.3 \times 10^4$</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND = not done.

Co-cultivation of B cells with RK13 monolayers

To examine the possibility that B lymphocytes could harbour RV in the absence of detectable replication, infected B cells were co-cultivated with cells known to be permissive to RV replication, in the form of sub-confluent monolayers of RK13 cells. Initially the B lymphocytes were purified from peripheral blood, but more recently, human tonsillar tissue has been used as a source to increase the yields obtained. Identical results have been found in each case.

Details of the protocol for co-cultivation are given in Methods. The results obtained with B cells after 5 days in culture with RK13 cells are compared with those obtained from mock-infected or infected mixed lymphocytes co-cultivated in an identical manner. However, in the case of the ML, cells were labelled and coverslips harvested on day 1 and day 3, instead of day 5 post-co-cultivation, as widespread cytopathology (c.p.e.) of the infected cultures was apparent by day 3. Although the infected B cell/RK13 co-cultivation did not exhibit such marked c.p.e., numerous foci of rounded cells could be seen by day 5.

The results of microfocus assay of the culture media are shown in Table 2. Infectious virus could readily be detected in both the co-cultivations of infected ML and B cells although the titres in the latter were 20- to 50-fold less. Similarly, indirect fluorescence assay of the coverslips using anti-rubella prepared in rabbits clearly demonstrated the presence of RV cytoplasmic antigens in both infected B cell and ML co-cultivations (Table 2). In the case of the ML co-cultivations, the fluorescence was widespread and involved >80% of the cells remaining in the monolayer on day 3, by which time 10 to 20% of the cells had detached. However, in the B cell co-cultivations, fluorescence was still localized in discrete foci by day 5 and only on day 8 post-co-cultivation was gross c.p.e. apparent.

Analysis of RV polypeptide synthesis in the RK13 co-cultivations is shown in Fig. 5. High backgrounds of host synthesis are present but most RV polypeptides including (p109), p75, p66, p50, p46, p40 and p32.5 can be detected. No qualitative differences were found in the polypeptides identified in ML or B cell co-cultivations; however, much greater amounts of p66 are present in the ML cultures. This polypeptide accumulates late in infection and is believed to be a precursor of one or more structural virus proteins (Chantler, 1979).

DISCUSSION

The present study has shown that human peripheral blood lymphocytes can support the replication of RV and has identified the T cells as the sub-population in which this occurs. No active replication was found in the B population but RV could be rescued from these cells by co-cultivation with RK13 cells suggesting that B cells or monocytes, or both, are capable of harbouring the virus. To rule out the possibility that virus was in fact being rescued from a small number of contaminating T cells, infectious centre assays were performed on B cell and monocyte populations and these indicated that 10 to 15% of the cells...
harboured infectious virus, while the percentage of contaminating rosetting cells was only 1 to 2%. However, this does not pinpoint the B cell as the site which harbours the virus, as only 70 to 60% of the cells in this population have been characterized as possessing SIg markers.

Several viruses have been shown to replicate selectively in T or B lymphocytes. Vesicular stomatitis virus (VSV); Newcastle disease virus and poliovirus are restricted to T cells (Bloom et al. 1976), Epstein-Barr virus and cytomegalovirus to B cells (Jondal & Klein,
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1973; Olding et al. 1975), while other viruses, such as measles virus (Joseph et al. 1975) and herpes simplex virus (Rinaldo et al. 1978) have been shown to replicate in both cell types when activated with a suitable mitogen. Although rescue of virus from the population normally non-permissive to virus replication has not previously been reported, Bloom et al. (1976) found that uptake of VSV into both T and B cells did occur, and by studying the infection of B lymphoblastoid cell lines they found that 13S and 26S mRNA species were synthesized and that the point of restriction appeared to be the synthesis of 42S virion RNA. Unstimulated mixed lymphocytes were also non-permissive to VSV replication until activated, and they concluded that B cells might also become permissive if a suitable B mitogen could be found. This may also be true of RV in our system.

Comparison of intracellular polypeptide synthesis in mixed lymphocytes and RK13 cells infected with RV has not indicated that any gross differences exist in the RV polypeptides produced. No evidence was found for a series of early proteins nor for partial expression of RV polypeptides in the non-permissive B cells. However, cellular protein synthesis, which is unaffected in RK13 cells, is greatly inhibited in the lymphocytes by 72 h p.i., a factor which has simplified the detection of RV proteins in these cells and eliminated the need for hypertonic labelling conditions. This inhibition of host cell function is in agreement with the substantial depression of mitogenic stimulation found after RV infection of lymphocytes and, together with the ease of detection of RV polypeptides, suggests that a high proportion of lymphocytes in the culture support virus replication after high multiplicity infection in vitro. This conclusion was also reached by Simons & Jack (1968) who found that the normal stimulation of up to 85% of lymphocytes could be abolished after in vitro infection. In contrast they found that although RV could be rescued from lymphocytes of congenitally-infected infants, these lymphocytes gave a normal response to PHA, suggesting that only a small percentage of cells was involved in persistent infection. In view of the ability of B population cells to harbour RV in the absence of detectable replication, shown here, it is possible that these cells are a site of long-term persistence of the virus (such as in congenital rubella or rubella panencephalitis) when some form of host restriction of virus replication must be present.

The authors thank Theresa Sapp and Theresa Yang for their excellent technical assistance. This project was supported by the Arthritis Society, Canada.

REFERENCES


*(Received 12 February 1980)*