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UNIVERSITY OF ALBERTA

REPLICATION OF RUBELLA VIRUS IN VERO CELLS AND PERIPHERAL BLOOD MONONUCLEAR CELLS

by

© YUN ZHENG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE IN VIROLOGY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA, CANADA

Spring, 1990

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DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: 1990

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled REPLICATION OF RUBELLA VIRUS IN VERO CELLS AND PERIPHERAL BLOOD MONONUCLEAR CELLS submitted by YUN ZHENG in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN VIROLOGY.

.....

Supervisor

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.....

Date: October 31, 1989

Dedicated to my parents and my friends

ABSTRACT

The interaction between rubella virus (RV) and human peripheral blood mononuclear cells (PBMC) has been suggested to abrogate the host immune function which may facilitate virus persistence. The scope of this study was to characterize the replication of rubella virus in peripheral blood mononuclear cells and its subpopulations.

In the initial studies on rubella virus replication in Vero cells, the virus was shown to replicate efficiently under the experimental condition used, and expressed the typical characteristics of wild type rubella virus previously described in the literature.

The permissiveness of PBMC and its subpopulations was investigated. Mitogen stimulation was required for productive viral infection in unfractionated PBMC. Among these cells, monocyte derived macrophages were the principal cells to support productive viral infection, as did the T cell enriched population to a limited degree. However, the mitogen stimulated B cell enriched population and unstimulated PBMC failed to produce infectious progeny virus, and the block was at the virus assembly or the release stage.

In addition, immunofluoresence and infectious center assays revealed that only a small percentage of PBMC and subsets were involved in persistent infection with rubella virus, and the time course of viral RNA and protein synthesis in Vero

cells and PBMC were similar. However, the efficiency of viral replication in PBMC and their subsets was much lower than that in Vero cells.

Also viral infection could depress the responsiveness of PBMC and their subpopulations to mitogens, and the inhibition was not due to a decreased viability of cells. Thus, rubella virus may disrupt immune cell function at the level of the monocyte, T cell and B cell. This virus-cell interaction under non-cytopathic conditions may provide a site for long-term persistence of rubella virus and subsequent adverse manifestations.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor, Dr. R. Marusyk, who has taught me the first steps of science. His expert guidance, support and patience made this thesis possible.

My sincere gratitude also extends to Dr. Aimo Salmi, for his expert advice and help in the viral immunology field and his generous support during the thesis work.

My warmest gratitude goes to Dr. D. L. J. Tyrrell, chairman of the Department, whose encouragement and support have been invaluable.

Special thanks go to Linda Chui, for her enthusiastic and experienced teachings and advice on technique.

Help on molecular biology techniques from Dr. K. Suryanarayana and photographic assistance from Mr. San Vinh, are appreciatiated.

In addition, I would like to thank Dr. D. Tovell and Ms. Kate Agopsowicz for revision of the text, and I would also like to thank all the other members in the Department for creating a helpful atmosphere to work in.

I would like to thank my parents and my friends for their continuous understanding and support.

Financial assistance provided by the Faculty of Graduate Studuies and Research, University of Alberta is acknowledged and I would like to take this opportunity to thank Canada.

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LIST OF ABBREVIATIONS

BSA	- bovine serum albumin
CPE	- cytopathic effect
CS	- calf serum
DEAE	- diethylaminoethyl
DNA	- deoxyribonucleic acid
EIA	- enzyme immunoassay
FCS	- fetal calf serum
FITC	- fluorescein isothiocyanate
HCMV	- human cytomegalovirus
Ig	- immunoglobulin
IL-2	- interleukin-2
KD	- kilodaltons
LPS	- lipopolysaccharides
m.o.i.	- multiplicity of infection
mRNA	- messenger ribonucleic acid
OD	- optical density
PBMC	- peripheral blood mononuclear cells
PBS	- phosphate buffered saline
pfu	- plaque forming unit
PHA	- phytohemagglutinin
p.i.	- post-infection
PWM	- pokeweed mitogen
RNA	- ribonucleic acid
RV	- rubella virus
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electrophoresis
SN	- supernatant fluids
SRBC	- sheep red blood cells
VSV	- vesicular stomatitis virus
V	- volt
w/v	- weight per volume
w/w	- weight per weight

1. INTRODUCTION

1.1 General introduction

Rubella virus causes an endemic and epidemic illness worldwide in distribution. The disease, resembling measles, is characterized by rash, lymphadenopathy and low grade-fever. Usually it does not result in serious consequences. As rubella seemed to be a mild illness of children, it did not receive much attention prior to 1941 (Horstmann, 1986) when an Australian ophthalmologist, Dr. N. Gregg, observed that pregnant women who became infected could spread the virus to the fetus (Gregg, 1941). If this occurs within the first trimester of pregnancy, the infection may cause congenital cataracts, deafness, heart disease and mental retardation in infants (Dulbecco and Ginsberg, 1988).

Since this mild disease can have disastrous consequences, it is very important to successfully immunize the target populations. Since 1969, several strains of effective, live-attenuated vaccine have been available, such as HPV-77 (Parkman, 1966), Cendehill (Pietermans and Huygelen, 1967) and RA 27/3 (Plotkin, 1967). Vaccination has markedly reduced the incidence of rubella (Bart et al., 1985). No large epidemics have occurred since 1970 (CDC, 1984). In United States 0.1 case/100,000 population was reported in 1988. The incidence of rubella has declined by more than 99% since 1969, the year rubella vaccine was licensed (CDC,

1989). However, RV is still widespread and endemic in many areas where people remain unvaccinated such as Latin America, Africa, Asia (Assaad and Ljungars-Esteves, 1985; Mingle, 1985), and the People's Republic of China, which has more than one fifth of the world's population (Wannian, 1985). Even in countries such as the USA, 10 to 20% of women of child-bearing age are still susceptible (Dulbecco and Ginsberg, 1988), in spite of an advanced immunization program.

Successful vaccination enhances both the humoral and cellular immunity against RV. However, the effectiveness of current vaccines is still far from perfect. The vaccine-induced antibody levels tend to be lower and to decline more rapidly than do those acquired as a result of natural infection (Hortmann et al., 1985). When challenged with either RA 27/3 vaccine or wild virus, inapparent reinfections can be readily induced in all vaccinees whose antibodies have declined to low or undetectable levels (Schiff et al., 1985). It has also been reported that several congenital rubella syndrome (CRS) infants were born to mothers who had seroconverted after receiving Cendehill vaccine seven to eight years previously (Enders, 1985). Failed rubella immunizations in adults are correlated with immunosuppression and persistence of rubella virus in peripheral blood mononuclear cells (Ganguly et al., 1985; Cunningham and Fraser, 1985; Tingle et al., 1985a). Prolonged arthritis,

neurological sequelae and chronic rubella viremia also developed in some vaccinees (Tingle et al., 1985b). Therefore, study of the virus is clinically important in the light of its capacity to induce congenital disease and its ability to cause persistant infection.

1.2 The properties of the virus

Rubella virus is the only species recognized so far in the Rubivirus genus of the Togavirus family. The name was derived from the Latin word 'Rubi' which means reddish (Matthews, 1982). Other genuses included in the Togaviridae are: Alphavirus (type species: Sindbis virus); Flavivirus (type species: Yellow fever), and Pestivirus (type species: mucosal disease virus).

1.2.1 Morphology

By electron microscopy, rubella virus appears as roughly spherical particles, measuring 50-70 nm in diameter. The outer surface of the particle is a plasma membrane-derived envelope with spike-like surface projections composed of the virus-specific glycoprotein E1 (63 KD) and E2 (47 KD). The envelope encloses an icosahedral nucleocapsid core which is built from multiple copies of the virus "c" (capsid) protein. A single-stranded, positive polarity viral genomic RNA (3,000 KD) resides inside the icosahedral core (Waxham and Wolinsky, 1983; Oker-Blom et al., 1983).

1.2.2 Chemical and physical properties

The buoyant density of rubella virions has been determined in several density gradient systems. Using CsCl gradients, values of 1.085 (Russel et al., 1967), 1.12 (Thomsen et al., 1968), and 1.32 g/cm^3 (Amstey et al., 1968) have been determined. In sucrose gradients, virion densities of 1.18 to 1.19 g/cm^3 have been obtained (Furukawa et al., 1967a, b; Sediwick and Sokol, 1970; Liebhaber and Gross, 1972).

The variability of the values obtained with CsCl gradients probably reflects the fragility and sensitivity of the virus to degradation (Oker-Blom, 1984a). However, Bohn and van Alstyne (1981) suggested that the differences in the buoyant density of RV recorded in the literature might simply reflect variations in the proportion of defective interfering (DI) particles and standard virus, and the history of the virus stocks used by each laboratory.

The virus is relatively heat labile. At 37°C, in 2% serum, 50% of the infectivity remains after 1 h (Parkman et al., 1964). All infectivity is lost within 30 min at 56°C (Fabiyi et al., 1966). The virus can be stored at -60°C for many years. However, infectivity may be lost rapidly at intermediate temperatures (-10°C to -20°C) (Parkman et al., 1964). Incubation at pH's below 6.8 or above 8.1 results in rapid loss of infectivity (Chagnon and Laflamme, 1964)

1.2.3 Immunological properties

Only a single antigenic type of RV has been detected and no serologic relationship exists between rubella and any other known virus. Minor biological variations that have been identified in different strains of rubella virus are not reflected in antigenic variation of the virus (Best and Banatvala, 1970; Fogel and Plotkin, 1969) The spike-like projections of the RV envelope glycoproteins E1 and E2 are responsible for the elicitation of immunity in the host (Green and Dorsett, 1986). Detailed immunological analysis using monoclonal antibodies revealed that the E1 glycopolypeptide was associated with both hemagglutination inhibiting (HI) and neutralization (NT) antibody activities (Green and Dosett, 1986; Waxham and Wolinsky, 1985). Recently, three epitopes which react with hemagglutination-inhibiting and neutralizing antibodies have been located between amino acids 245-285 in the predicted amino acid sequence of the E1 protein (Terry et al., 1988). Such information enhances the possibility for development of a synthetic vaccine. The E2 protein has been suggested to be either less exposed or less immunogenic than the E1 protein, although it was found that one epitope of E2 has neutralization activity (Trudel et al., 1985; Green and Dorsett, 1986).

1.3 The structure of the virus

1.3.1 Proteins

The study of the RV structural proteins has been complicated by the difficulty in obtaining purified virus proteins against the background of cellular protein. This is evident by the differences in the species of the proteins identified and the corresponding molecular weights reported in the literature (Table 1).

The virion consists of three major structural proteins; the envelope glycoproteins E1 (55-63 KD) and E2, and capsid protein C (30-38 KD) (Toivonen et al., 1983; Oker-Blom et al., 1983). The E2 protein migrates either as a diffuse band or as two to three separate bands in SDS-PAGE. Oker-Blom et al. (1983) detected two bands and designated them as E2a (47 KD) and E2b (42 KD). The heterogenicity of the E2 protein was due to the differences in glycosylation of the polypeptide. Both the E1 and E2 proteins are glycosylated and associated with the viral envelope (Ho-Terry and Cohen, 1982). The C protein is nonglycosylated and rich in arginine residues which are believed to participate in the binding of the 40S genome RNA to form the nucleocapsid (Oker-Blom et al., 1984; Clarke et al., 1987). These three proteins are coded in the viral genome. The order of translation of RV structural proteins is NH_2-C-E2-E1-COOH (Oker-Blom, 1984b).

Table 1. Rubella virus structural proteins observed by different authors.

authors	size (KD)	methods
Chantler and Tingle (1980)	75, 50, 46-40, 32.5	SDS-PAGE Autoradiography
Oker-Blom et.al., (1983)	58, 47-42, 33	SDS-PAGE Autoradiography
Toivonen et.al., (1983)	62-60, 51-44, 35	Immunoprecipitation
Bowden and Westaway (1984)	59, 48-43, 34	Immunoprecipitation
Waxham and Wolinsky (1985)	62, 54-47, 38	Immunoprecipitation

1.3.2 RNA

The genome of RV is a single stranded RNA of positive polarity about 10,000 nucleotides in length (Oker-Blom et al., 1984a) sedimenting at 38S to 40S with a size about 3,000 KD (Sedwick and Sokol, 1970; Hovi and Vaheri, 1970).

1.3.3. Defective interfering (DI) particles

Rubella virus readily generates DI particles under conditions by which DI RNAs are generated by other viruses. Several groups of investigators were able to detect DI RNAs in their infecting stock of RV (Norval, 1979; Bohn and van Alstyne, 1981; Terry et al., 1985; Frey and Hemphill, 1988). DI particles were increased after high multiplicity passage, accompanied by a decrease in virus yields and infectivity (Bohn and van Alstyne, 1981; Terry et al., 1985). Northern hybridization analysis revealed that the amount of intracellular genomic RNA relative to the amount of DI RNA species decreased dramatically (Frey and Hemphill, 1988), indicating that DI RNA species have the ability to interfere with replication of the genomic RNA. However, in the case of RV, it was found that RV DI paticles were generated during persistent infection, but their presence was not necessary for initiation of persistence (Frey and Hemphill, 1988). To understand what role the DI particles play in establishment and maintenance of RV persistence needs further investigation.

1.4. Replication of the virus

1.4.1 Virus penetration

Acid-induced conformational change of the spike protein of enveloped viruses was believed to be part of the mechanism by which viral genetic information enters the cells via the acidic intracellular vesicles (Katow and Sugiura, 1988). Although the penetration and uncoating processes of RV have not been examined in detail, the similarity of the penetration process of RV to that of Semliki Forest Virus, another virus in Togaviridae, was suggested (Kobayashi, 1978; Väänänen and Kääriäinen, 1978). Recently, Katow and Sugiura (1988) found that fusion of RV infected cells and irreversible conformational change of viral envelope polypeptides were induced by low pH, and during the process the virus acquires liposome-binding activity. These results suggest that rubella virions fuse with the host cell membrane in a similar way as some other enveloped viruses.

1.4.2 Replication strategy of structural proteins of rubella virus

In addition to the 40S genomic RNA, the RV infected cells contained a subgenomic 24S RNA species of roughly 3,500 nucleotides in length which corresponds to the 3' one-third of the genomic RNA and serves as messenger for translation of virus structural proteins. Both RNA species are

polyadenylated and have 5'-terminal caps (Oker-Blom et al., 1984). The strategy for the expression of RV structural proteins was first proposed by Oker-Blom et al.(1984) and later confirmed by cDNA sequencing and expression (Clarke et al., 1987). Translation of the 24S subgenomic RNA produces a 110 KD precursor polyprotein (p110) which is post-translationally processed to yield structural proteins, C (capsid), E2a, E2b and E1, with size of 33, 47, 42 and 58 KD (see Fig. 1).

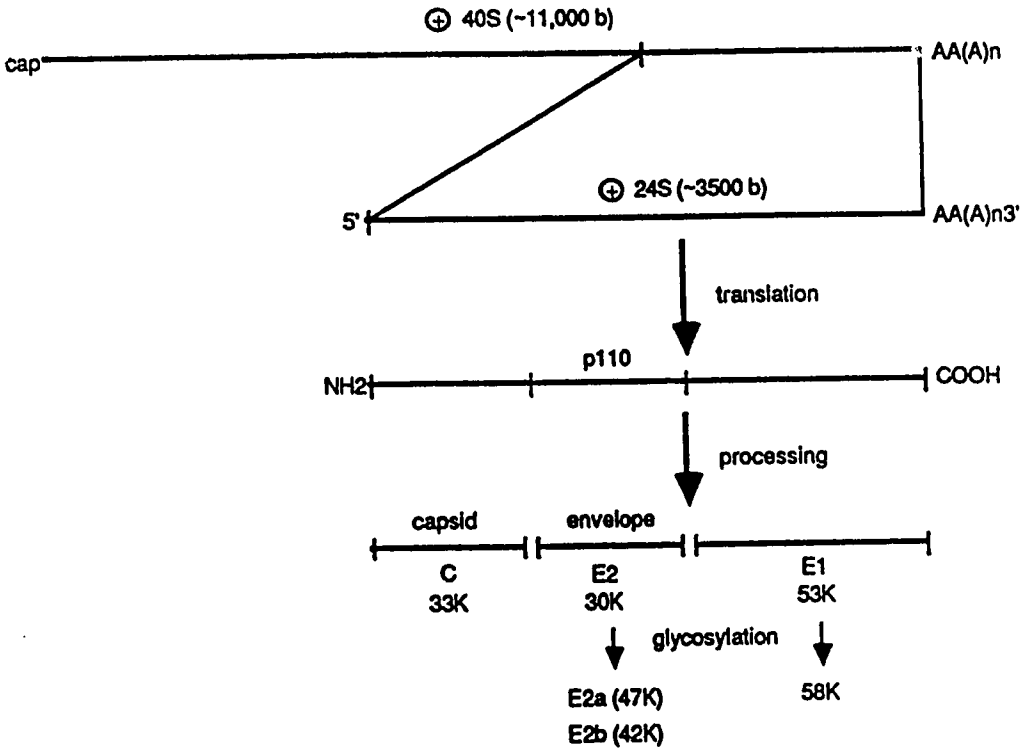
1.4.3 Virus maturation

It was proposed that rubella virions mature via a budding process from host cell membranes. The nucleocapsid core assembly occurs at the same time as host membrane deformation in early budding (Murphy 1980). However, the details of virion maturation are not clear.

1.4.4 Time course of virus-specific macromolecular synthesis during infection

In infected Vero cells, the rate of virus production rises rapidly and reaches a peak level at 48 h p.i. The number of living cells at 96 h p.i. was around 70%-80% of that in mock-infected cells. The structural proteins are first detected intracellularly at 16 h p.i. and simultaneously reach a maximal rate of synthesis which is maintained through 48 h p.i. Cells expressing virus proteins detected by immunofluorescence using rabbit anti-RV polyclonal antiserum

Figure 1. The replication strategy of structural protiens of rubella virus. (adapted from Oker-Blom, 1984a)


⊕ 40S (~11,000 b)
cap
AA(A)n
⊕ 24S (~3500 b)
5'
AA(A)n3'
translation
p110
NH2
COOH
processing
capsid
envelope
C
33K
E2
30K
E1
53K
glycosylation
E2a (47K)
E2b (42K)
58K

are first observed at 16 h p.i. In infected Vero cells both genomic and subgenomic RNA species are detectable at 12 h p.i. and reach their highest synthetic rate at 26 h p.i. (Hemphill et al., 1988). In infected BHK cells, the peak rate of RNA synthesis was reached as early as 30 h p.i. (Sedwick and Sokol,1970). No study has been reported on the rate of virus-specific RNA and protein synthesis in PBMC and its subpopulations.

1.4.5 RV-cell system

Considerable evidence has accumulated over the years suggesting that there is a participation of host factor(s) in the viral synthetic processes of RNA viruses, such as encephalomyocarditis (Dmitrieva et al., 1979) and polio virus (Dasgupta et al., 1980; Morrow et al., 1985). This was also observed in the case of RV (Nakhasi et al.,1988). When actinomycin D, was incorporated into RV infected cultures at the early stage of virus intake (<4 h), the synthesis of viral RNA and protein was abolished. This observation suggested that host cell DNA-directed synthesis of the cellular factor(s) is important for the initiation of replication of RV.

Interferons are groups of regulatory proteins that affect the replication of a wide range of RNA and DNA viruses (Stewart, 1979; Kirchner and Schellekens, 1985). However, the inhibitory ability depends on the type of cell and the

virus studied (Lengyel, 1986). Nakhasi et al. (1988) found that α- and γ-interferons exerted inhibition of RV protein synthesis at both transcriptional and translational levels.

On the other hand, RV has the capacity to inhibit cell macromolecular synthesis, although the degree of inhibition depends on the host cell type. In infected Vero cells, virus had no effect on cell RNA synthesis but a low degree of inhibitory effect on cell protein synthesis (Hemphill et al., 1988). In BHK-21 cells, 30-50% of total protein synthesis was inhibited by 72 h p.i. (Payment et al., 1975) An inhibitory effect on cell protein synthesis was also found in human peripheral blood lymphocytes, but not in infected RK-13 cells (Chantler and Tingle, 1980)

1.5 Rubella cDNA clones

Several types of rubella cDNA clones have been isolated and characterized. A cDNA clone encoding the precursor of all three structural proteins (E1, E2 and C) has been constructed. When the plasmid was introduced into COS cells, all three proteins were expressed (Clarke et al., 1988). Nucleic acid hybridization using a cloned rubella cDNA probe to detect RNA synthesis of rubella virus has been used with clinical material (Ho-Terry et al., 1988), as well as in RV infected Vero cells (Hemphill et al., 1988). This has been described as a sensitive, rapid and specific technique, especially for the detection of DI particles (Hemphill et

al., 1988). However, use of this technique with RV infected PBMC and its subsets has not yet been reported.

1.6 RV persistence

There have been various reports in the literature (Mims, 1982) in the last few years outlining the persistant infection caused by DNA and RNA viruses under both clinical and experimental conditions. However, the mechanisms leading to persistent infections are still poorly understood.

RV readily establishes a persistent infection in every cell line which has been studied, including BHK-21 (Kilburn and Wezel, 1970), BSC-1, Hela (Stanwick and Hallum, 1974), RK-13 (Svedmyr, 1965), rabbit embryonic chondrocytes (Smith et al., 1973), LLC-MK_2 (Norval, 1979), and Vero cells (Maassab and Verpnelli, 1966). It has been reported that RV can grow for at least 50 sub-cultures in the LLC-MK_2 cell line with no loss in ability to release infectious virus (Maassab et al., 1964).

Rubella persistence *in vivo* has been associated with some forms of chronic arthritis. Persistent arthritis is considered more common after vaccine administration than after natural rubella (Spruance et al., 1972). Virus has also been recovered from peripheral blood lymphocytes of both naturally infected and vaccinated patients with arthritis lasting for as long as ten years. These patients were undergoing reinfections, not primary infections (Chantler and

Tingle, 1982b). It was suggested that partial immunity in the form of low antibody levels and some cell-mediated immunity, can prevent acute viraemia but cannot eliminate the virus. This weak immune response tends to favor persistence, a state in which virus replication can be periodically reactivated. Production of virus could result in the formation of virus-antibody immune complexes, which in turn might initiate a localized inflammatory response in the joints (Chantler et al., 1982). Indeed, after immunization it was found that levels of immune complexes in patients with arthritis symptoms were higher than in those who remained symptom-free (Verder et al., 1986)

In the congenital rubella syndrome, virus specific cell-mediated immunity is impaired, especially in children with the so-called "late onset rubella syndrome" (LORS). LORS following maternal infection in the first trimester of pregnancy is associated with a series of organ-specific autoimmune diseases (Rubenstein, et al., 1982; Clarke et al., 1984). The clinical picture is dominated by virus persistence and disseminated infection with progressive meningoencephalitis as a prominent feature. Verder et al. (1986) found that LORS was associated with immune complex disease and defective cytotoxic effector cell function. When they first studied patients at the age of five months, there was a remarkable absence of suppressor/cytotoxic T cells, and decreased activity of K and NK cells. Removal of cell-bound

immunoglobulin and immune complexes tended to improve K and NK cell function *in vitro*. Plasma exchange transfusion carried out in patients at 9 months of age resulted in normalization of cytotoxic effector cell function and cessation of viremia. The authors proposed that a large amount of rubella antigen and anti-rubella IgM complexes coating the cytotoxic-effector cells would interfere with their function, causing delayed virus elimination.

For a thorough understanding of the immunobiology and pathogenesis of rubella persistence, there is no doubt that a further understanding of the relationship between RV and lymphoid cells will be required.

1.7 Interaction of rubella virus with cells of the lymphoid system.

Human peripheral blood mononuclear cells, if stimulated with a mitogen such as phytohaemagglutinin (PHA) have been shown to support the replication of a number of viruses *in vitro*, such as mumps virus (Duc-Nguyen and Henle, 1966), measles virus (Berg and Rosenthal, 1961), vesicular stomatitis virus (Edelman and Wheelock, 1966), vaccinia virus (Miller and Enders, 1968) and herpes simplex virus (Nahmias et al., 1964). Moreover, a number of viruses have been isolated from lymphocyte populations in both acute and persistent infection, including measles (Horta-Barbosa et al., 1971) and cytomegalovirus (Lang and Noren, 1968).

Therefore, it is likely that lymphocytes serve as one of the prefered reservoirs of virus replication and persistent infection. The virus suppresses lymphocyte responsiveness to antigen or mitogen, and thus protects itself from immune attack.

In some cases, only one cell type within the PBMC is susceptible to infection. For example, Epstein-Barr virus is known to infect only a small percentage of B cells (Menezes et al., 1976), whereas herpes simplex and mumps viruses exhibit a preference for T cells only (Kirchner, et al., 1977; Flerscher and Kreth 1982). In other cases, such as with measles virus, T and B cells and monocytes can be all infected (Sullivan et al., 1975).

In the case of RV, replication in human PBMC *in vitro* has not been investigated extensively. Little is known about the mechanism of pathogenicity and the behaviour of the virus in those immune cells. Direct evidence of virus replication in human PBMC *in vitro* is extremely limited, although the virus has been isolated from lymphoid tissues after acute natural infection (McCarthy et al., 1963; Chantler and Tingle, 1982a), or vaccination (Buinovici-Klein and Cooper, 1979), and also during persistent infection in the case of congenital rubella (Simons and Jack, 1968).

There have been three publications on RV replication in PBMC and subsets *in vitro*. Chantler and Tingle (1980)

reported that PBMC and T cells stimulated with PHA or PWM supported high levels of RV replication ($\geq 1 \times 10^6$ pfu/ml), although a low level of virus replication could be maintained by unstimulated PBMC ($\approx 1 \times 10^3$ pfu/ml). PWM-stimulated B cells did not support the replication of RV, although the virus could be recovered from these cells by co-cultivation with rabbit kidney (RK_{13}) cells. Inadequate mitogen stimulation of B cells has been suggested. In addition, van der Logt et al. (1980) found that stimulated PBMC could support the replication of of RV (Gilchrist stain) to a limited degree ($\approx 1 \times 10^3$ pfu/ml). The monocyte derived macrophages were the principal cell type to support virus replication ($\approx 5 \times 10^3$ pfu/ml). The susceptibility of macrophages to RV infection increased from day 1 (2.5%) to day 4 (26.5%). Recently, the HPV/DE5 RV vaccine strain has been shown to infect B cells productively and $CD4^+$ and $CD8^+$ T cells when stimulated by the appropriate mitogens (Barth and Chantler, 1987).

Inhibition of PHA-stimulation by live RV was first demonstrated in lymphoid cells from babies with congenital rubella syndrome (Olson et al., 1967). Subsequently, the phenomenon was studied following vaccination against rubella. It was reported that inhibition induced by attenuated virus strains is generally less intense than that induced by virulent strains (McMorrow et al., 1974).

The possibility of lymphokine production was investigated. Maller and Soren (1978) reported that in the case of the attenuated strain RA27/3, heat- or UV-inactivated supernatants from primary cultures infected with live-RV inhibited the PHA stimulation of secondary uninfected cultures. In contrast, no inhibition was obtained by supernatants from primary cultures inoculated with UV-inactivated virus, in which a similar degree of antigen-specific lymphocyte proliferation was recorded. The result indicates that interferon may be generated in supernatants from live-RV infected primary cultures, as interferon has been shown to inhibit PHA stimulation of lymphocytes (Lindahl-Magnusson et al., 1972).

1.8 Objectives of this study

RV has been isolated from peripheral blood mononuclear cells following naturally acquired and vaccine-induced infections (Heggie and Robbins, 1964; Buimovici-Klein and Cooper, 1979; Chantler and Tingle, 1982a; O'Shea et al., 1983). Accumulated evidence suggests that it is highly likely that RV infected PBMC play a role in the pathogenesis of infection.

However, it is still not clear which sub-population(s) of PBMC support virus replication. Chantler and Tingle (1980) demonstrated that, *in vitro,* RV replicated preferentially in the T cell population, although virus could be recovered from

both B cells and monocytes by co-cultivation with rabbit kidney cells. More recently, Barth and Chantler (1987) found that RV could replicate in both $CD4^+$ and $CD8^+$ T cells as well as B cells. This contrasted with the findings of van der Logt and colleagues (1980) who demonstrated that monocyte-derived macrophages rather than T or B cells supported replication of the virus. In addition, O'Shea et al. (1988) reported that, *in vivo,* RV antigens were expressed more frequently on the monocyte than on lymphocyte populations.

Moreover, little is known about the molecular biology of RV in the human lymphoid cell system. Thus, the objectives of this study were:

1. To investigate whether RV (Therien strain) would replicate in human peripheral blood mononuclear cells;

2. To determine which sub-population of PBMC supports the replication of RV;

3. To characterize RV replication in PBMC and subsets; to define the sequential rate of virus specific macromolecular synthesis.in comparison with the Vero cell line;

4. To detect the effect of RV infection on PBMC and its subset function.

2. MATERIALS AND METHODS

2.1 Cultivation of Vero cells

Vero cells (African green monkey kidney, ATCC CCL 81) were used for propagation of stock virus and plaque assays, as well as control cells positively infected with RV, throughout this study.

Cells were routinely cultured in Blake bottles with modified Eagle's minimal essential medium (MEM) (Gibco Laboratories Inc., Burlington, Ont.) supplemented with 1% glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin sulfate and 3% heat-inactivated calf serum (Flow Laboratories, Mississauga, Ont.) adjusted to pH 7.4 with 0.75% sodium bicarbonate. Cells were passaged every 4 days and cultured at 37°C.

2.2 Isolation of PBMC by centrifugation with Ficoll-Pague™

Human PBMC were isolated from heparinized venous blood from healthy adult donors (buffy coat from Red Cross Blood Transfusion Center, Edmonton, Alberta) according to Böyum (1968).

Phosphate buffered saline (PBS, pH 7.4) diluted whole blood (1/10) was carefully layered onto Ficoll-Paque™ gradients (Pharmacia Fine Chemicals, Piscataway, NJ) and

centrifuged at 400 x g for 30 min at 20°C. The PBMC (lymphocytes and monocytes) layer was collected by aspiration and washed three times in PBS to remove contaminating platelets, Ficoll-Paque™ and plasma. The yield of viable cells was measured by counting using Trypan Blue exclusion.

Cells were adjusted to 5 x 10^6/ml in RPMI 1640 medium (Gibco Laboratories, Burlington, Ont.) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco Laboratories, Burlington, Ont.), 100 IU/ml penicillin, 0.1 mg/ml streptomycin sulfate and cultured at 37°C with 5% CO_2. In some circumstances, 10 µl/ml of phytohemagglutinin-P (PHA) (Sigma Chemicals, St. Louis, MO, USA) was added, as this concentration of PHA causes maximal stimulation of PBMC under our culture conditions.

2.3 Enrichment of monocytes by adherence or Percoll™ gradient centrifugation

i) adherence method

PBMC in RPMI 1640 with 10% FCS were incubated in polystyrene tissue culture petri dishes (Corning Glass Works, Corning, NY) at a concentration of 5 x 10^6/ml for 1 h at 37°C. Non-adherent cells were removed, and the plates were washed three times with PBS. The adherent cells were incubated in PBS plus 2 mM EDTA at 4°C for 15 min and scraped off with a rubber policeman. After three washes with PBS, the cells were suspended in supplemented RPMI and counted.

ii) Percoll™ discontinuous density gradient

The discontinuous density gradients of Percoll™ used for monocyte purification consisted of 5 different density layers with discrete interfaces (Fluks, 1981).

A stock solution of Percoll™ (90%) was prepared by mixing 90 ml undiluted Percoll™ (Pharmacia, Fine Chemicals, Piscataway, NJ) with 10 ml of Hanks' balanced salt solution (10x). The pH of the stock solution was adjusted to 7.4 with 1N HCl. Discontinuous density gradients were obtained by successive layering of Percoll™ solutions of decreasing densities. The amount of stock Percoll™ solution plus RPMI (10% FCS) used to obtain the desired densities is listed.

Gradient(ml)	Density(g/ml)	90% Percoll™(ml)	RPMI(ml)
2	1.060	10	10.5
2	1.062	10	9.8
2	1.066	10	8.6
2	1.070	10	7.6
5	1.080	10	5.4

5 x 10^7 PBMC were suspended in 5 ml of the Percoll™ solution (d=1.080) and placed in the bottom of a 15 ml centrifuge tube, 2 ml aliquots of Percoll™ solution with respective densities of 1.070, 1.066, 1.062, and 1.060, were carefully layered on top of each other. After centrifugation for 30 min at 400 x g at room temperature, the top band was collected and the cells were washed three times in PBS. The

purity of monocytes was determined by MMA monoclonal antibodies (Hanjan et al., 1982).

2.4 Enrichment of T cells by fractionation on Nylon wool column

After two adherence cycles as described in section 2.3, the non-adherent cells were passed through a nylon wool column (Julius et al., 1973) to deplete the B cells.

Nylon wool in LP-1 Leuko Pak Leukocyte filters (Fenwal Laboratories, Morton Graves, Illinois) was soaked overnight in Milli-Q™ prepared water and then rinsed with three changes of water to remove any toxic material which would decrease survival of the cells. Aliquots of about 0.6 gram of dried nylon wool were packed into 10 ml plastic syringes to the 7 ml mark. After sterilization by autoclaving, the sterilized nylon columns were soaked in supplemented RPMI 1640 at 37°C for 30 min. Two ml of non-adherent cell (1 x 10^8/ml) suspension were added to the nylon wool and the fluid was allowed to go through the column. The syringe was capped and incubated at 37°C for 1 h. Twenty ml of medium were passed through the column and the eluate containing unbound cells was collected and centrifuged at 400 xg for 5 min. The resulting pellet was a T cell enriched population. The purity of the cells was determined by anti-leu-4 monoclonal antibody (Kan, et al., 1983).

2.5 Enrichment of B cells by sheep blood cell rosetting.

T cells form rosettes when incubated with sheep red blood cells (SRBC), and this is enhanced by treating the SRBC with AET-solution (2-aminoethylisothiouronium bromide hydrobromide) (Sigma Chemical Co., St. Louis, MO. USA).

An 8% AET solution was made in PBS and adjusted to pH 8.2 with 5 M NaOH. Four ml of this AET solution was mixed with SRBC and incubated 15 min in 37°C. SRBC were then washed with PBS until no hemoglobin colour remained, then resuspended in supplemented RPMI medium to a final concentration of 3%. A non-adherent cell suspension (5 x 10^6/ml) was mixed with AET treated SRBC in equal amounts, incubated at 37°C for 15 min and centrifuged. The cell pellet was incubated on ice for 30 min, resuspended, and layered on Ficoll-Paque™ as described in the isolation of PBMC.

The T cells sedimented to the bottom with the SRBC allowing the B cells at the interface to be collected, and examined by staining of surface immunoglobulin (SIg).

2.6 Cultivation of stock virus and infection of cells

The Therien strain of RV was a gift of Dr. Aimo Salmi, Department of Medical Microbiology and Infectious Diseases, University of Alberta. The virus had previously been plaque-

purified three successive times at the University of Turku, Finland.

Roller bottles were precoated with FCS for 2 h prior to seeding with about 2 x 10^7 Vero cells. RV at a multiplicity of infection (m.o.i.) of 0.1 to 0.01 was used to infect the sub-confluent cell monolayer at day 2. After an adsorption period of 2 h at 37°C, cells were washed twice with PBS to remove the unadsorbed virus. Fifty ml of MEM containing 5% FCS and 25 mM HEPES (N-2-hydroxyethyl-1-piperazine-N'-2-ethane sulfonic acid) (Sigma Chemical Co. St. Louis, MO. USA) buffer, pH 7.4. was added. At day 3 post-infection (p.i.) the medium was replaced with 30 ml of fresh medium. Supernatant fluids (SN) were harvested at days 5 and 7, clarified by centrifugation at 2,000 x g for 30 min at 4°C, and stored in aliquots at -70°C. A titer of 2.5 x 10^7 pfu/ml was obtained by standard plaque assay of the stock virus.

PBMC and subsets in suspension were exposed to RV at a m.o.i. of 3 to 5 for 2 h at 37°C with frequent shaking. Cells were then washed and resuspended in fresh supplemented RPMI medium.

2.7 Purification of virus

About 1.5 L SN harvested from infected Vero cells was clarified by low speed centrifugation (2,000 x g, 30 min, 4°C) and concentrated by a Millipore Minitan™ concentrator (Millipore, Mississauga, Ont.) to about 100 ml. Concentrated

SN was layered on a 20 and 60% (w/w) sucrose step gradient in TNE buffer (0.05 M Tris, 0.001 M EDTA, 0.15 M NaCl, pH 7.4) and centrifuged for 90 min at 24,000 rpm (Beckman SW 27 rotor) at 4°C. The material at the interface was collected, diluted and then layered on a linear 30% to 60% sucrose gradient, and centrifuged at 24,000 rpm (Beckman SW 27 rotor) for a minimum of 4 h. The virus band was collected as 2 ml fractions, dialyzed against PBS and analyzed by enzyme immunoassay (EIA).

2.8 Preparation of Vero cell lysates

Vero cell monolayers were washed in PBS and scraped off with a rubber policeman. Cells were freeze-thawed five times and homogenized in a Dounce-type homogenizer. Cell debris was removed by centrifugation at 2000 x g for 30 min and the SN was centrifuged at 6000 x g for 30 min at 4°C. The pellet was resuspended in PBS, and the amount of protein was determined by Lowry protein assay (Lowry et al., 1951), and stored in aliquots at -70°C. The material was sonicated before being used as control antigen in EIA.

2.9 PBMC and subsets proliferation assay

The optimal doses of mitogen required to stimulate PBMC and monocyte enriched populations were determined by titration. PBMC or monocytes (1×10^5 cells/0.1 ml/well) were seeded in 96-well round bottom plates. Different concentrations of PHA or lipopolysaccharride (LPS) (Sigma

Chemical Co. St. Louis, Missouri) were added. Cells were pulsed with 14.8 KBq/well (0.4 µci/well) [^{3}H]-thymidine (74 GBq/mmmol (2Ci/mmol), ICN Biomedicals, Canada Ltd., Montreal, Quebec) 18 h before harvest and then cultured at 37°C in a 5% CO_2 environment and harvested with a multiple cell harvester (Skatron, Inc., VA) onto glass wool filters. After washing and lysing the cells with water, the filters were dried and counted in a liquid scintillation spectrometer. The results were expressed as mean counts per min (CPM) ± standard deviation (SD) of six wells.

To assay the effect of RV on PBMC and subsets blast transformation response, the optimal concentration of mitogen was added to both mock-infected and infected cultures, cells were pulsed, harvested, and uptake of [^{3}H]-thymidine was measured as described above.

2.10 Plaque assay

Titers of infectious virus were determined by a standard plaque-forming assay on Vero cells. Virus-containing SN were serially diluted 10-fold and 0.1 ml of each dilution was added in duplicate to Vero cell sub-confluent monolayers in 12-well plates (Flow Laboratories Inc. Mississauga, Ont.). The number of virus plaques on duplicate wells usually differed by less than 10%.

For the detection of cell-associated virus, infected cells were freeze-thawed three times in 0.5 ml of PBS and

then passed through an 18 gauge needle three times. Vero cells were infected for 2 h at 37°C with frequent shaking. The monolayers were over-laid with 2% carboxymethyl cellulose (CMC) (Sigma Chemical Co. St. Louis, Missouri) in MEM supplemented with 100 IU/ml of penicillin, 0.2 mg/ml of streptomycin sulfate, 2 mM glutamine, 0.75% sodium bicarbonate and 1.5 mM HEPES buffer, and incubated at 37°C in a 5% CO_2 environment. Plaques were stained with 0.2% crystal violet in 5% formaldehyde and 10% ethanol for 2 to 4 h and counted.

2.11 Infectious center assay

Infected cells were washed and vigorously pipetted to make single-cell suspension. Cells were diluted 10-fold to concentrations ranging from 10^2 to 10^6 cell/ml and duplicate 0.1 ml aliquots of each dilution were added to sub-confluent Vero cell monolayers in 9.6 cm^2 wells of six-well plates (Becton Dickinson Co., Lincoln Park, NJ). After adsorption for 2 h at 37°C, the cells were overlaid with 2% CMC in MEM, as described in Section 2.10 and incubated for 7 days. The results were expressed as pfu/10^2 cells.

2.12 Generation and characterization of antiserum against purified virus antigen

2.12.1 Immunization of a rabbit

A New Zealand white rabbit (Health Sciences Laboratory Animal Services of the University of Alberta) was pre-bled and then injected subcutaneously with 0.5 mg of purified RV antigen in Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, MI). Two weeks later, a serum sample was collected and the rabbit was boosted with 0.25 mg of the purified virus in incomplete Freund's adjuvant. The boosts were repeated at two week intervals up to ten weeks. The final injection was given intravenously and the rabbit was terminally bled 7 days later. Test bleeds were collected to moniter serum antibody levels by a plaque neutralization test and an EIA.

2.12.2 Plaque neutralization assay

Rabbit anti-RV preimmune serum as well as immune serum was diluted twenty-fold in supplemented MEM. A pre-determined amount of RV that contained 50 PFU was added to each dilution of serum and incubated at 37°C for 1 h. A 0.2 ml aliquot of each sample was inoculated onto each of two monolayers of Vero cells grown in 9.6 cm^2 wells of six-well plates (Becton Dickinson Co., Lincoln park, NJ). After adsorption for 2 h at 37°C, the cells were overlaid with 2%

CMC in MEM and incubated as described above. Titers were expressed as reciprocals of the highest immune serum dilution giving a 50% reduction in the number of plaques obtained with preimmune serum.

2.12.3 Enzyme immunoassay

Microtiter plates (Linbro, Flow Laboratories, Inc. McLean, Virginia, USA) were coated with either 1 µg/well of purified virus or 1.5 µg/well of Vero cell lysate overnight at 4°C. The free binding sites on the plates were blocked with EIA diluent (PBS supplemented with 0.5% bovine serum albumin (BSA), 0.5% Tween-20 and 0.1 mM merthiolate) for 2 h at room temperature. The plates were washed three times with a washing solution (PBS supplemented with 0.1% Tween-20) and incubated with rabbit preimmune or immune serum for 1 h at 37°C. After a further washing as above, horseradish peroxidase (HRPO) conjugated anti-rabbit IgG (Cedarlane Laboratories, Hornby, Ont.) was added and incubated for another hour at 37°C. Substrate solution (*O*-phenylenediamine plus 7 µl of 30% hydrogen peroxide in 10 ml of 0.1 M citrate buffer, pH 5.5) was added and incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 0.05 ml/well 5 N HCl and the plates were read with a Titertek Multiskan™ photometer (Eflab, Helsinki, Finland) at OD_{492}.

2.12.4 Pre-adsorption of antiserum

The antiserum generated was absorbed two times prior to use. Firstly, PBMC were washed in PBS, dried onto a petri dish and fixed in methanol at -20°C for 10 min to expose the internal non-specific binding sites. Antiserum was then added to the cell monolayer and incubated for 2 h at 37°C. Secondly, the antiserum was simply mixed with PBS washed PBMC, shaken at 37°C for about 2 h and then the mixture was centrifuged. The SN thus obtained was considered to be adsorbed antiserum.

2.13 Immunofluorescence

2.13.1 Surface immunofluorescence

Ascites fluid obtained from a mouse hybridoma cell line (MMA) was used to identify monocytes by indirect surface immunofluorescence. Cells (1×10^6) in Eppendorf tubes were incubated for 30 min on ice with a predetermined optimal dilution of MMA in a volume of 0.2 ml. After two washings with PBS supplemented with 1% CS, fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added, incubated and washed as above.

FITC conjugated goat-$F(ab')_2$ fragment polyvalent anti-human Ig (Cedarlane Laboratories, Hornby, Ont.) was used to identify B cell populations by direct surface

immunofluorescence. Anti-leu-4 FITC (Becton Dickinson, Mountain View, CA, USA) was used to identify T cell population.

Immune-stained cells were suspended in PBS buffered glycerol and mounted onto microscope slides. The percentage of surface marker positive cells was calculated by combining fluorescence and phase contrast microscopic observations. At least 100 cells were counted.

2.13.2 Cytoplasmic immunofluorescence

To detect intracytoplasmic viral antigens, Vero cells were grown on multi-well slides, (Flow Laboratories, Inc. Mississauga, Ont.), incubated in petri dishes and infected at a m.o.i. of 5 as described in section 2.6. At each time p.i., the slides were washed with cold PBS and fixed for 10 min in acetone at -20°C. Mock- or infected PBMC and subsets were washed two times with PBS supplemented with 1% CS and centrifuged onto microscope slides (Cytospin 2, Shandon Instruments, Sewickley, PA, USA) and fixed in acetone at -20°C.

Pre-immune or immune serum prepared previously was added and the slides were then incubated for 1 h at 37°C in a humidity chamber. After extensive washing, the slides were stained by affinity isolated FITC-conjugated anti-rabbit IgG (Cedarlane Laboratories, Hornby, Ont.).

Pre-adsorption of serum or dilution of antibody in PBS plus 1% CS was found to decrease non-specific fluorescence to some extent.

2.13.3 Double immunofluorescence

Double-labeling was carried out in PBMC after 2 days incubation with RV. The presence of both surface markers and intracytoplasmic viral antigens was determined by combining the above procedures. After surface staining, labeled cells were centrifuged onto microscope slides and fixed with acetone. The cells were then stained for intracytoplasmic RV antigen with a rabbit polyclonal antiserum. After incubation and washing, cells were further incubated for 1 h in biotinylated anti-rabbit IgG (Cedarlane Laboratories, Hornby, Ont.) followed by streptavidin-Texas red (Sigma Chemical Co., St. Louis, MO, USA) for another 30 min. Slides were mounted and observed with a Leitz microscope with a selective filter system for counting fluorescein (surface staining) and Texas red (cytoplasmic staining). About 100 cells were counted.

Human RV positive serum (Virology Division, Provincial Laboratory of Public Health, Edmonton, Alberta) and rhodamine-conjugated anti-human IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) were used in some experiments and neither was found to be as satisfactory as rabbit serum or the Texas red system used in our conditions.

2.14 Western blot

Mock- or infected cells (2 x 10^6) were incubated in 0.5 ml of lysate buffer (1% SDS, 1 mM phenylmethyl sulphonyl fluoride, 1% trasylol, 0.1% Triton X 100 in Tris-HCl, pH 6.8) on ice for 20 min and then passed through a 23 gauge needle five times. Cell debris was removed by centrifugation. Samples were boiled for 5 min in Laemmli sample buffer (Laemmli, 1970) and applied to SDS-polyacrylamide gels, containing 5% stacking gel and a 10% separating gel, for electrophoresis (SDS-PAGE). After electrophoretic separation, proteins were electrophoretically transferred to nitrocellulose as described by Towbin et al. (1979). The nitrocellulose was blocked in 3% BSA in TBS (10 mM Tris-HCl, pH 7.4, 0.9% NaCl) for 1 h at 37°C and rinsed with TBS. Rabbit anti-RV preimmune or immune serum was added and incubated for 1 h at 37°C, with shaking. After washing in TBS, a biotinylated anti-rabbit IgG as second antibody (Cedarlane Laboratories, Hornby, Ont.) was added and incubated for another hour. Binding of antibodies was detected by subsequent incubation with streptavidin-HRPO complex (Cedarlane Laboratories, Hornby, Ont.) for 1 h and stained with colour development solution (4-chloro-1-naphthol) (Bio-Rad Laboratories, Richmond, Calif.) in the presence of H_2O_2. The reaction was stopped by immersing the blot in water. The blot was then air dried.

2.15 Radio-immunoprecipitation

To radiolabel intracellular proteins, mock- or RV-infected (m.o.i. of 5) PBMC and subsets were washed with PBS and pulsed for 16 h in RPMI containing one tenth of the normal concentration of methionine plus 1.85 MBq/ml (50 µci/ml) of [^{35}S]-methionine (37 TBq/mmol (1000 Ci/mmol), ICN Biomedicals Canada Ltd., Montreal, Quebec) before harvesting. The labeled cells were washed with PBS and chased for 30 min with supplemented MEM. After another washing, cells were lysed in NET buffer (0.4 M NaCl, 0.05 M Tris (pH 8.0), 0.005 M EDTA, 1% NP-40 containing 100 µg/ml Trasylol), for at least 30 min, and the solution was clarified by centrifugation. Aliquots (100 µl)of cytoplasmic extracts were incubated with 200 µl of a 10% suspension of protein A-Sepharose CL-4B (Sigma, Chemical Co., St. Louis, Missouri) in PBS for 12 h at 4°C to reduce the level of nonspecific binding of radiolabeled proteins to the adsorbent. The adsorbent was removed by centrifugation and the SN was incubated with 20 µl of rabbit preimmune or immune serum for 1 h at 37°C. A 10% suspension of protein A (200 µl) was added and incubation was continued at 4°C for 12 h with shaking. The adsorbent was collected by centrifugation, washed four times in NET buffer, boiled 5 min and subjected to SDS-PAGE under the conditions described for the Western blot. For autoradiography, the SDS-gel was soaked in Amplify™ (Amersham, Oakville, Ont.) for 30 min followed by soaking in 3% glycerol to prevent

cracking during the subsequent drying step. The dried gel was exposed to Kodak X-omat AR X-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen for 2-3 days at -70°C.

2.16 Generation of virus specific probes

A cDNA clone pKTH345 containing RV E1 and E2 genes was kindly provided by Dr. Ralf Pettersson (University of Helsinki, Finland). The insert, estimated to be about 2 kilobases in size, was cloned into the *Pst* I site of pBR322 using the dC-dG tailing procedure (Vidgren et al., 1987).

2.16.1 cDNA clone amplification

i) Competent E.coli cell preparation

A single colony picked from an *E.coli* HB101 culture was inoculated into 100 ml LB (Luria-Broth) medium and vigorously shaken at 37°C for about 3 h to allow the bacteria to reach the log phase of growth. The cells were harvested by centrifugation at 4000 xg, at 4°C for 10 min and resuspended in 10 ml of solution I (10 mM MOPS/RbCl (morpholinopropane sulfonic acid/rubidium chloride), pH 7.0). Cells were reharvested and incubated in solution II (100 mM MOPS, pH 6.5, 50 mM $CaCl_2$ and 10 mM RbCl) for 15 min on ice. After centrifugation, the cells were resuspended in solution II. Competent cells thus obtained were used within 24 h.

i) Transformation

Sixty ng of pKTH345 were incubated with 200 µl competent cells in ice for 45 min and heat shocked for 2 min at 42°C. One ml LB medium was added to the cells and the suspension was incubated at 37°C for 1 h. One hundred µl or 200 µl fractions were evenly spread on LB plates containing 10 µg/ml tetracycline (Tet). The plates were incubated at 37°C overnight in the dark.

iii) Mini-prep of plasmid DNA by the alkaline lysis method.

Single colonies of HB101 cells grown on Tet/LB plates were picked and inoculated into 3 ml of LB with 30 µg Tet. The cultures were shaken at 37°C overnight. Approximatlly 1.5 ml of the culture was transferred into an Eppendorf tube and centrifuged for 1 min. The cell pellet was resuspended and incubated in 100 µl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) and freshly added lysozyme (at a concentration of 4 mg/ml) at room temperature for 5 min and then lysed with freshly prepared solution II (0.2 N NaOH, 1% SDS) for 5 min followed by neutralization with 150 µl of 3 M sodium acetate, pH 4.8, for 5 min on ice. After centrifugation, the SN were transferred to a fresh Eppendorf tube and extracted with equal volumes of phenol-$CHCl_3$ and $CHCl_3$ alone. Between each extraction, the aqueous phase was separated from the organic phase by centrifugation and transfered to a fresh tube. Two volumes of cold 95% ethanol

were added to the aqueous solution and the plasmid DNA was precipitated at -20°C overnight. The plasmid DNA was pelleted by centrifugation and rinsed with cold 70% ethanol. The pellet was dried and resuspended in 30 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

To analyze the isolated plasmid, 10 µl of the above sample was digested with 5 U of *Pst* I as well as 1 µg of DNase-free RNase A in REact™ #2 buffer (BRL/GIBCO) at 37°C for 1 h. The sample was run on a 0.8% agarose gel in Tris-acetate (TAE) at 60 V for 2 h and stained with ethidium bromide (1 ug/ml). The bands were visualized under UV light.

iv) Large scale preparation of plasmid DNA

LB medium (1 L) was inoculated with a small fraction of the bacterial culture which had been confirmed to contain the plasmic pKTH345 by a mini-prep and subsequent restriction endonuclease analysis. The bacterial culture was allowed to grow at 37°C overnight with vigorous shaking. The cells were harvested by centrifugation and lysed with alkali using the method used in the mini-prep except that the volume of each solution was increased. After centrifugation, the pellet was suspended in TE buffer, pH 8.0, and then extracted extensively with phenol-chloroform for deproteinization. The plasmid thus obtained was subjected to digestion by three restriction endonucleases, including *Pst* I , *Kpn* I, and *Sal* I and analysed on a 5% polyacrylamide gel, buffered by 89 mM

Tris-boric acid and 2 mM EDTA (TBE), pH 8.0. λ DNA digested by *Eco* RI and *Hind* III was used as the standard. The bands were visualized by ethidium bromide staining.

2.16.2 Isolation of DNA fragments from agarose gel using DEAE membrane.

A DEAE membrane (Schleicher & Schuell Inc., Keene, NH) was pre-treated with 10 mM EDTA, pH7.6, for 10 min, 0.5 M NaOH for 5 min, rinsed and stored at 4°C in Milli-Q™ water. The plasmid obtained in the large scale preparation was digested with *Pst* I and run on 0.8% TAE agarose as described in section 2.16.1. After staining with ethidium bromide, a slit was cut in the gel at the leading edge of the DNA band to be extracted. A piece of the pre-treated membrane of the proper size was then inserted into the slit and the gel was electrophoresed again until the DNA had migrated onto the membrane. The DEAE membrane was then removed and rinsed in NET buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 8.0). To elute the DNA, the membrane was immersed in high salt NET buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 8.0) and incubated at 60°C for 1 h with occasional shaking. The membrane was removed and rinsed with 50 µl high salt NET, and the buffers were pooled and extracted with three volumes of water-saturated n-butanol to remove ethidium bromide. Finally, two and half volumes of ethanol were added and incubated at -20°C to precipitate the DNA. Twenty four hours later, the DNA was reprecipitated with 1/10 volume of 3 M

sodium acetate and 2 volumes of ethanol to remove residual NaCl.

2.16.3 Labeling of RV specific DNA probes.

Labeling of RV specific DNA probes was carried out by two methods.

The random priming procedure of Feinberg and Vogelstein (1983) was carried out with a non-radioactive DNA labeling and detection kit (Boehringer Mannheim, Dorval, Quebec). In brief, DNA was labeled by randomly primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate (dUTP). After hybridization to the target DNA, the hybrids were detected by enzyme immunoassay using an antibody-conjugate and enzyme-catalyzed colour reaction.

The second method produced [^{32}P]-labeled probes by nick translation (Boehringer Mannheim, Dorval, Quebec) to a specific activity of 1.2×10^8 cpm/µg DNA.

2.17 Extraction of total RNA from Vero cells, PBMC and subsets.

Efforts were made to prevent any contamination by RNases, including baking glassware, diethyl pyrocarbonate (DEPC) treatment of water and buffers and wearing disposable gloves throughout all the operations.

Among several RNA isolation methods, the guanidine thiocyanate method (Macdonald et al., 1987) was found to be a successful procedure for isolation of RNA from Vero cells or PBMC. Therefore this method, with some modifications, was used.

Vero cells or PBMC and subsets (2×10^7 in a siliconized Eppendorf tube) were lysed in 1 ml of a solution containing 4 M guanidium thiocyanate, 0.1 M Tris-HCl, pH 7.5, 1% 2-mercaptoethanol and 0.5 ml of sodium lauryl sarcosinate on ice. The lysis solution was passed through a 23 gauge needle five times to shear the nuclear DNA and reduce the viscosity of the solution. Cell debris was removed by centrifugation in a microfuge for 15 min and 0.5 ml of 2 M potassium acetate, pH 5.5, as well as 0.8 ml of 1 M of acetic acid were added. The solution was then mixed with 0.5 ml of 95% ethanol while vortexing and stored at -20°C overnight. The precipitate was collected by centrifugation for 10 min. The pellet was resuspended in 1 ml of 7.5 M guanidine-HCl containing 10 mM EDTA, pH 7.0, and reprecipitated by mixing with 0.25 ml of 2 M potassium acetate, pH 5.5, and 2.5 ml of ethanol at -20°C overnight. The pellet was again collected by centrifugation for 10 min and resuspended in 0.05 ml of 10 mM EDTA, which was subsequently extracted with 1.2 ml of chloroform-n-butanol (4:1 v/v). The aqueous phase was removed and the organic phase as well as the interface were extracted twice with 10 mM EDTA, pH7.0. The extracts were

combined and then extracted by phenol-chloroform. Two hundred and fifty µl of 2 M sodium acetate, pH 7.0, and 750 µl ethanol were added and the solution was stored at -20°C overnight. The RNA precipitate was collected by centrifugation for 15 min, dried and resuspended in 25 µl of DEPC-treated water. The amount of RNA was estimated spectrophotometrically at 260 nm. For storage, the RNA samples were precipitated by adding 0.1 volume of 2 M sodium acetate, pH 7.0, and 2.5 volumes of ethanol at -20°C.

2.18 Slot blot detection with enzyme-labeled probes

RNA samples extracted from mock- or infected Vero cells (10 µg) or from PBMC and subsets (20 µg) were denatured in 50% deionized formamide and 6% formaldehyde in a total volume of 100 µl at 65°C for 15 min. The samples were diluted with 6x SSC (1x SSC is 150 mM NaCl, 15 mM Na citrate, pH 7.0) to a volume of 200 µl and spotted onto pretreated nitrocellulose paper (soaked in Milli-Q™ water for 5 min and 2x SSC for 15 min) using a slot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.). The membrane was air dried and baked overnight at 60°C. Prehybridization was carried out at 37°C for 1 to 4 h in a solution containing 50% formamide, 3% blocking reagent (Boehringer Mannheim, Dorval, Quebec), 0.2% SDS and 0.1% (w/v) N-lauroyl-sarcosinate in 5x SSC. Hybridization was carried out in the above solution plus 20 ng/ml digoxigenin-labeled probe and 50 mM Tris-HCl, pH 8.0, at 37°C overnight. Washings were carried out in 2x SSC, 0.1%

SDS at 68°C for 15 min and followed by 0.1x SSC, 0.1%SDS for 15 min at 68°C.

The blots were blocked with 0.1 % blocking reagent at 37°C for 1 h and colour development was carried out according to the package insert (Boehringer Mannheim, Dorval, Quebec). In brief, the blots were washed and incubated with alkaline phosphatase-conjugated antibody and the hybridized probes were then visualized by incubation with 5-bromo-4-chlor-3-indolyl phosphate and nitroblue tetrazolium (NBT).

2.19 Northern blot detected by [^{32}P]-labeled probes

RNA samples extracted from mock- or infected Vero cells (20 µg) or from PBMC (30 µg) as well as RNA markers (*E.coli* 16S-23S ribosomal RNA) were denatured in 50% deionized formamide, 6% formaldehyde and 1x MOPS/EDTA at 65°C for 15 min and quickly chilled on ice. Samples were mixed with a dye mixture containing 0.1% bromophenol blue, 0.1% xylene cyanole, 35% Ficoll™, 0.5% SDS and applied to a 1% agarose gel containing 6% formaldehyde in 1x MOPS/EDTA buffer. Electrophoresis was carried out for 4 h at 100 V. The marker lanes were cut off and stained with ethidium bromide. Gels were rinsed in DEPC-H_2O three times, soaked in 50 mM NaOH for 15 min and neutralized with 0.1 M Tris-HCl, pH 7.5 for 30 min. Finally, the gels were equilibrated in 10x SSC for 30 min. The RNA was transfered to nitrocellulose in 20x SSC for

24 h using the method described by Maniatis (1982). The blot was rinsed in 6x SSC for 10 min and baked at 60°C overnight.

Prehybridizations were carried out at 42°C overnight in a solution containing 50% deionized formamide, 5x SSPE (1x SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA, pH 7.4), 5x Denhardt's solution (0.1% Ficoll™, 0.1% polyvinylpyrrolidone, 0.1% BSA) 5% dextran sulfate, 0.25 mg/ml denatured salmon sperm DNA. Hybridizations were carried out in the same solution as above except 0.1% SDS and 1x Denhardt's plus denatured [^{32}P]-labeled probes (10^6 to 10^7 cpm/ml). The blots were washed as described for blots hybridized with digoxigenin-labeled probes. Air dried blots were wrapped in Saran wrap and exposed to Kodak X-omat AR X-ray film with intensifying screens at -70°C.

3. RESULTS

3.1 Virus replication in Vero cells

In order to study the interaction between rubella virus (Therian strain) and human lymphoid cells, it was necessary to characterize the virus growth in Vero cells.

3.1.1 Cytopathic effect of rubella virus

According to Stanley and Plotkin (1964), the cytopathic effect (CPE) of RV begins with increasing refractility of focal areas, followed by rounding of the cells in the foci. The involved cells eventually detached from the monolayer. Fig. 2 shows the appearance of RV CPE in the system used.

Vero cells were infected at a m.o.i. of 0.1 and cultured in MEM with 2% FCS. The CPE could be observed at day 4 p.i., when some cells began to swell. By day 8, more cells became swollen and some cells detached from the monolayer.

The appearance of the CPE apparently depended on external conditions. Serum concentrations above 2% were found to depress CPE, as did the addition of vitamins or amino acids when changing culture medium. When medium was replaced at day 3 with medium containing an increased concentration of fetal calf serum (5%) the cell monolayer could last as long as ten days.

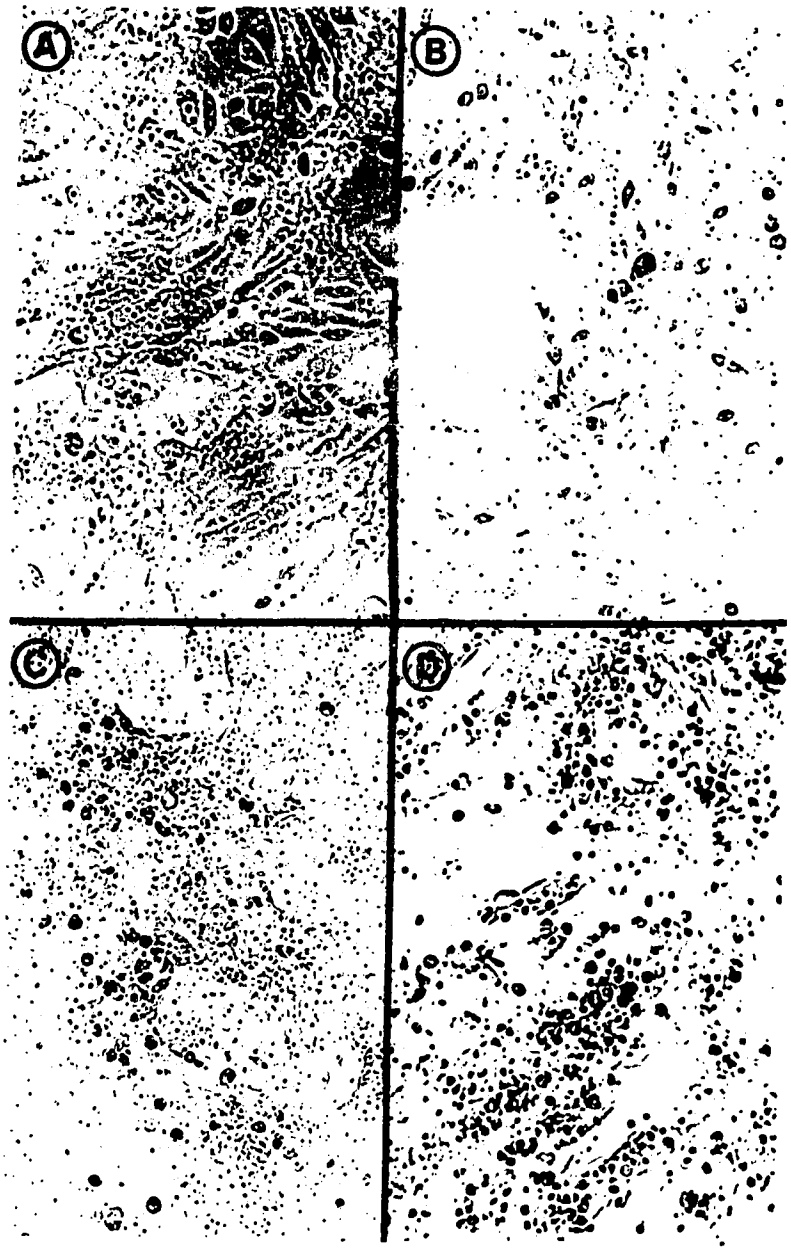
Figure 2. CPE of rubella virus on Vero cells. Vero cells were infected at a m.o.i. of 0.1 and cultured in MEM with 2% FCS at 37°C. Observations were carried out daily and photographs were taken using an Olympus inverted light microscope. Magnification 100x.

A: Mock-infected vero cell monolayer showing normal cell morphology.

B: 2 days p.i., only a few rounded cells (≈5%) can be seen.

C: 4 days p.i., about 45% cells have started to round up.

D: 8 days p.i., about 75% of the cells have rounded up showing extensive CPE.


A
B
C
D

These results demonstrated that the CPE of RV appeared much slower and to a lesser extent than that of some other virus under the same conditions, for example, measles virus destroys infected cells within four days p.i. (Dr. R. Marusyk, personal communication).

3.1.2 Optimal conditions for virus growth

RV replication was assayed by collecting the virus released from Vero cells infected at different m.o.i. at different times p.i., and measuring the infectious virus by plaque assay. The results are shown in Fig. 3.

The rate of virus production rose rapidly. By day 3 p.i., virus titers averaging 1 x 10^5 pfu/ml were obtained. Peak levels of pfu/ml were seen at day 5 p.i. Afterwards the rate of virus production decreased. By day 11, the virus titer declined to about 1 x 10^4 pfu/ml.

The highest virus titer (2.5 x 10^7 pfu/ml) was obtained at day 5 or 6 with an input m.o.i. of 0.1. Therefore, a m.o.i. of 0.1 was chosen for the cultivation of stock virus and used throughout this study.

The titers of virus preparations were determined by a standard plaque assay method on Vero cell monolayers grown in 12-well plastic tissue culture plates. The question of density of seeded cells in each well deserves comment. Table 2. shows the influence of cell density on virus production.

Figure 3. RV growth curves at different m.o.i. in Vero cells. Cells grown in large roller bottles were infected with RV at different m.o.i. as indicated. After adsorption for 2 h, cells were washed twice with PBS. A 30 ml amount of MEM containing 5% FCS and 25 mM HEPES (pH 7.2) was added. The infected culture fluid was havested and replaced with fresh medium at each time p.i. The virus growth curves from representative experiments are shown.

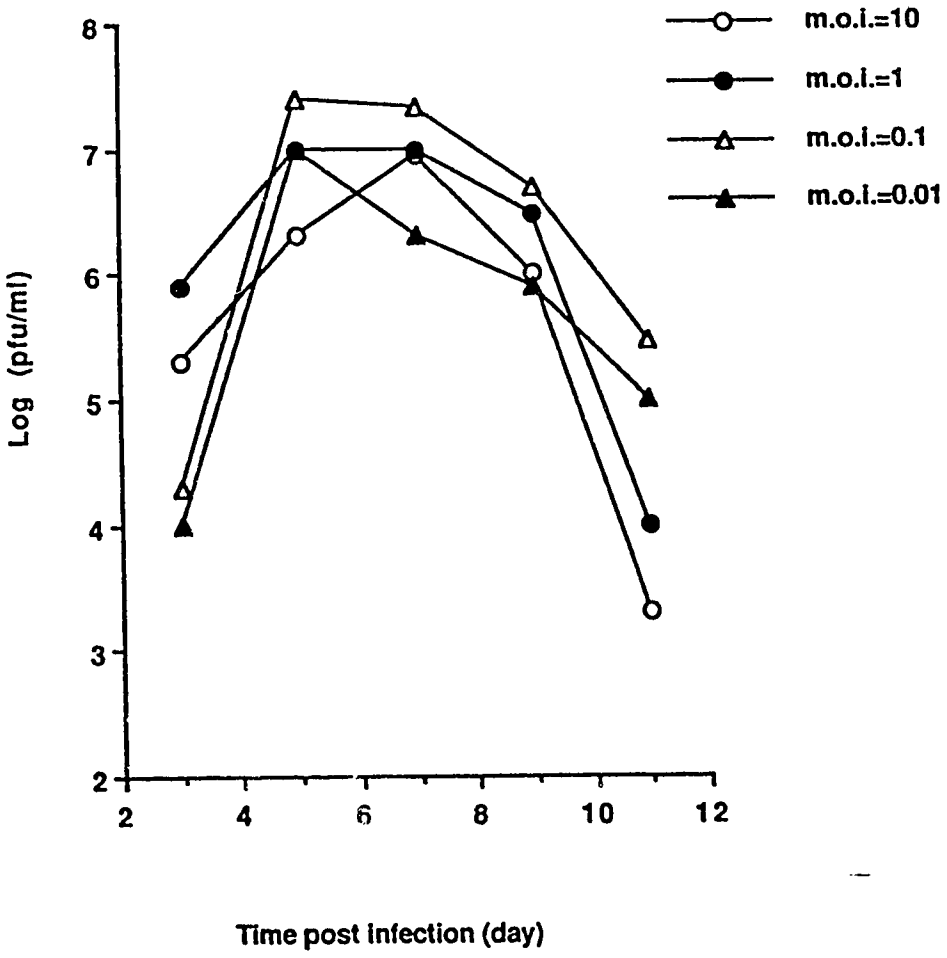

m.o.i.=10
m.o.i.=1
m.o.i.=0.1
m.o.i.=0.01
Log (pfu/ml)
8
7
6
5
4
3
2
2
4
6
8
10
12
Time post infection (day)

Table 2: The Effect of cell density on virus titer. Different numbers of Vero cells were seeded in 12-well plastic tissue culture plates. The cultures were inoculated 24 h later with a 10-fold dilution of supernatant fluids from infected cells RV titers were unknown. A minimum of 20 plaques were counted and the data represent the mean of two experiments.

Number of cells seeded (cells/well)	Virus titer (pfu/ml)
1.5×10^5	2.0×10^7
4.5×10^5	5.0×10^4

It is obvious that a subconfluent cell monolayer was more suitable for RV propagation. This might be due to the fact that low density cell cultures (about 1.5 x 10^5 cells/well) permit cell proliferation which, in turn, may better support virus replication.

3.1.3 Purification of virus

The RV particle is highly labile (Chagon and Laflamme, 1964; Parkman at al., 1964) which makes purification of the virus more difficult.

RV purifications were done mainly according to Pettersson et al. (1971) with some modifications as described in *Materials and Methods*. The results of EIA suggested that the majority of the fractions collected from a sucrose gradient contained RV antigens as shown in Fig. 4.

SDS-PAGE (see Fig. 5) showed that the purified virus contained three distinct bands with molecular weights of about 62 KD (E1); 42 KD (E2); and 35 KD (C), which agree with previously published data (see Table 1). It was also noticed that by this improved purification procedure, highly purified virus was obtained even though the harvests were done at a stage when CPE was extensive. Unsatisfactory results were observed previously by Oker-Blom (1984a) using the original procedure at the point of extensive CPE.

Figure 4. EIA titers of fractions of purified RV from sucrose gradient. A pre-determined optimal amount of purified RV was coated onto EIA microtiter plates and the RV positive or negative serum was serially diluted four-fold. Titers are expressed as the reciprocal of the $\log_2$ of the highest RV positive serum dilution to yield four times the counts of RV negative serum.

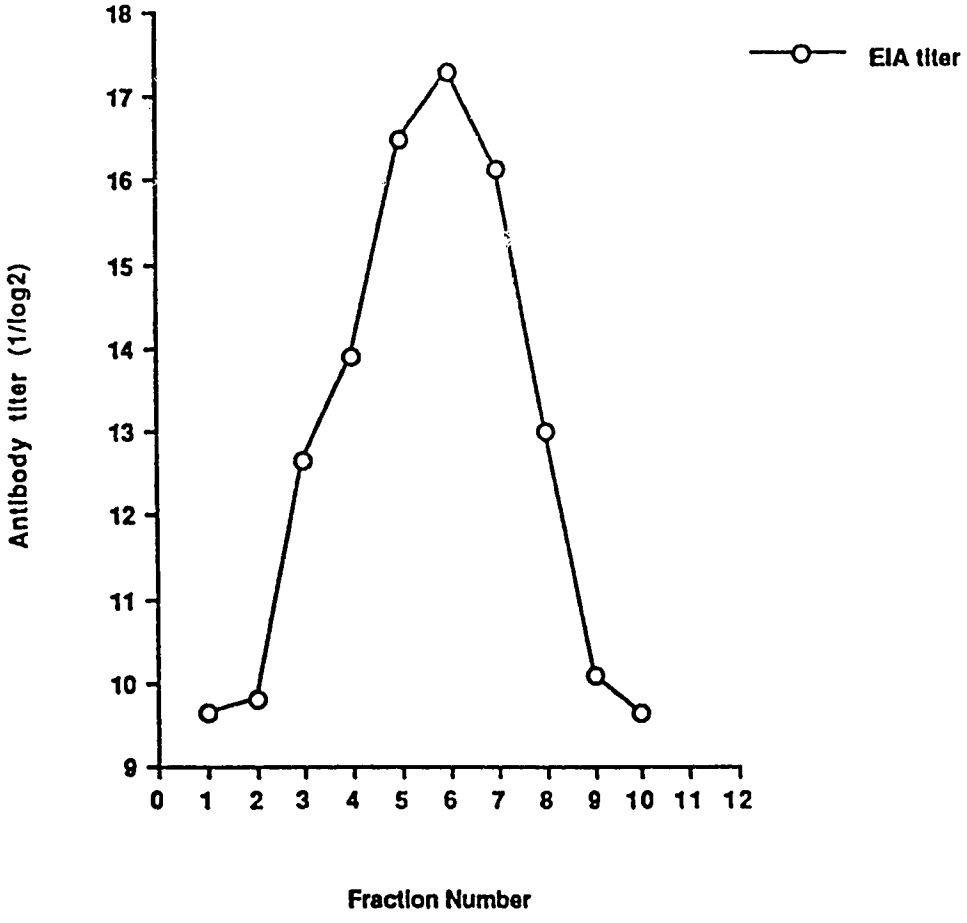

EIA titer
Antibody titer (1/log2)
18
17
16
15
14
13
12
11
10
9
0 1 2 3 4 5 6 7 8 9 10 11 12
Fraction Number

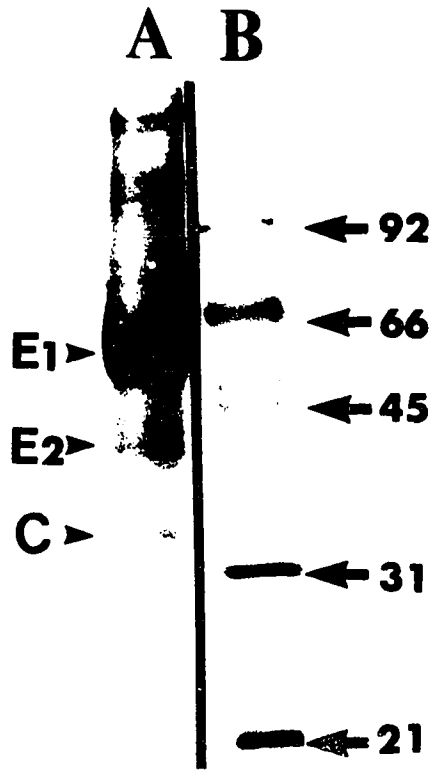
Figure 5. Purity of rubella virus as shown by SDS-PAGE and silver-staining. Virus was purified from infected cell supernatants as described in section 2.7. The product was solubilized in Laemmli sample buffer and analyzed by SDS-PAGE (5% stacking gel and 10% separating gel) in the presence of 2-mercaptoethanol. Silver-stain was down according to Bio-Rad bulletin 1089 with slightly modification.

A: purified virus.

B: molecular weight markers (KD).

E1 and E2: RV specific envelope glycoproteins.

C: RV capsid protein.


A
B
92
66
45
31
21
E1
E2
C

3.2 Production and characterization of antiserum against the purified rubella virus

Antisera to RV were generated by immunizing a rabbit with purified virus at two week intervals for up to two and half months. To detect the biological activity of the serum, a plaque neutralization test (NT) was used (see Fig. 6A). Titers were expressed as reciprocals of the highest immune serum dilution giving a 50% reduction in numbers of plaques from preimmune serum.

In addition, an EIA test against both purified virus antigen and uninfected Vero cell lysate was carried out (Fig. 6B). Data are expressed as the reciprocal of the $\log_2$ of the highest serum dilution yielding four times the counts (OD_{492}) of preimmunization serum (Milich and Mclachlan 1986).

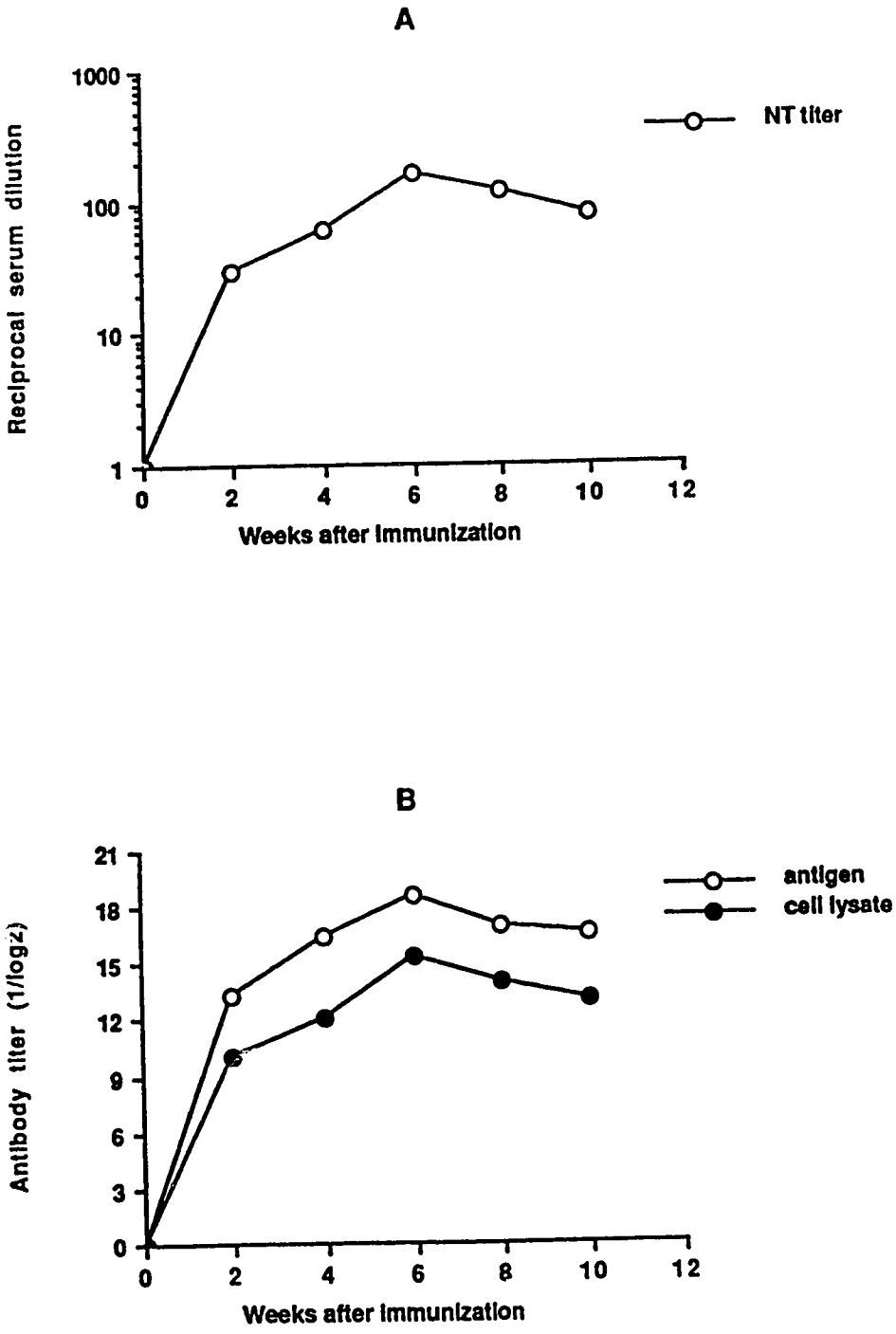
Both the NT and EIA results indicate that the specific antibody response against RV appeared by two weeks after the first injection. NT and EIA titers increased and reached a maximum at six weeks, then decreased gradually.

The quality of the antibody response is of interest. The response of antiserum to uninfected Vero cell lysate antigen (Fig. 6B) suggested that the non-specific "background binding" also increased concomitantly during the process of immunization, but the titers were relatively lower than those against purified RV antigen. The elicitation of a non-specific response indicated that some cellular components were present

Figure 6. Kinetics of antibody synthesis against purified rubella virus in rabbit. A rabbit was immunized at two week intervals. Sera were collected and analyzed by NT and EIA.

Figure 6A. Titers of antisera in plaque neutralization tests. Experimental procedures are described in section 2.12.2. NT titers were expressed as reciprocals of the highest immune serum dilution giving a 50% reduction in number of plaques counted in the presence of preimmune serum.

Figure 6B. EIA titers against purified RV antigen as well as uninfected Vero cell lysates. EIA microtiter plates were coated with either a pre-determined optimal amount of purified RV or uninfected Vero cell lysates (section 2.8), the preimmune serum or immune serum was titrated in four-fold dilutions.


A
1000
100
10
1
Reciprocal serum dilution
NT titer
0
2
4
6
8
10
12
Weeks after immunization
B
21
18
15
12
9
6
3
0
Antibody titer (1/log2)
antigen
cell lysate
0
2
4
6
8
10
12
Weeks after immunization

in the purified virus antigen that was inoculated into the rabbit.

Since antibodies against host cells might interfere with serological tests in various ways (Timbury, 1963), it was therefore necessary to absorb the serum with uninfected cells to reduce the nonspecificity. Fig. 7 shows the EIA titers of six week serum, before and after absorption with unfixed and fixed Vero cells. The results indicate that serum non-specific binding to the uninfected cell lysate is considerably decreased after absorption treatment. Therefore, pre-absorption of antiserum was done throughout this study.

The Western blot tests using purified virus antigen against both preimmune and immune serum were also done (see Fig. 8). The results demonstrated that serum obtained after six weeks of immunization reacted with all three virus structural proteins E1, E2, and C (Fig. 8, lane A). However, the preimmune serum showed no reactivity with purified virus antigen (Fig. 8, lane B). These results confirmed the production of specific rabbit anti-rubella polyclonal antibody.

3.3 Characterization of human PBMC and subsets

3.3.1 Characterization of subpopulations of PBMC

A monocyte-rich fraction was prepared by adherence to polystyrene plastic surfaces. The lymphocyte-rich fraction

Figure 7. EIA titers showing the effect of preabsorption of antiserum. Serum was incubated with both unfixed and cold acetone fixed Vero cells or PBMC at 4°C as described in section 2.12.3.

1' antigen: antibody response to purified RV antigen before serum absorption.

1' cell lysate: Antibody response to cell lysate antigen before serum absorption.

2' antigen: Antibody response to purified RV antigen after serum absorption

2' cell lysate: Antibody response to Vero cell lysate antigen after serum absorption.

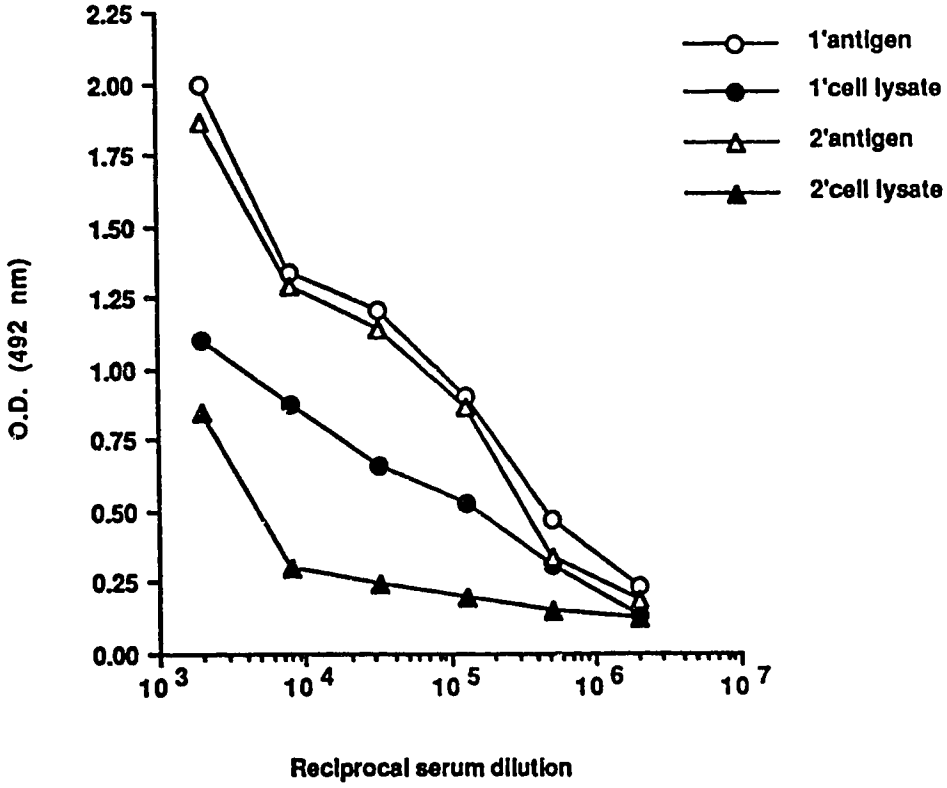
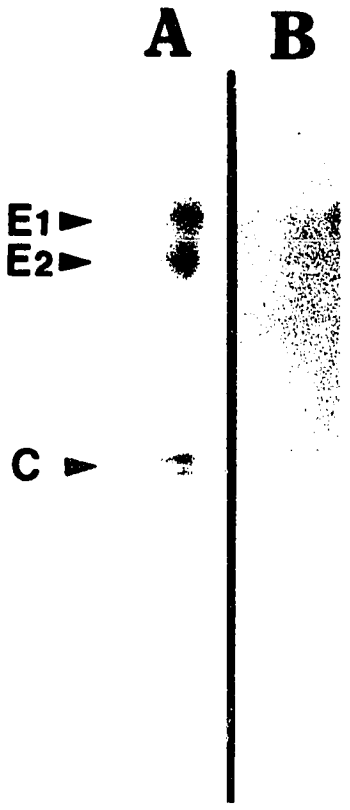

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
O.D. (492 nm)
10 3
10 4
10 5
10 6
10 7
Reciprocal serum dilution
1'antigen
1'cell lysate
2'antigen
2'cell lysate

Figure 8. The Western blot analysis of rubella virus specific rabbit serum. Purified RV antigen prepared previously was subjected to a 10% SDS-PAGE, and transfered to nitrocellulose paper. Western blot analysis was carried out using preimmune serum and immune serum, utilizing a biotinylated second antibody and streptavidin-peroxidase complexes.

Purified RV antigen detected with:

Lane A: immune serumm

Lane B: pre immune serum.


A
B
E1
E2
C

remaining in the supernatant was then either passed through nylon wool to obtain T cell enriched population or used for rosette formation with sheep red blood cells (SRBC) to obtain B cell enriched population. Monocyte enriched populations were also obtained by discontinuous Percoll™ gradient centrifugation.

The degree of purification of each subpopulation was analysed by surface immunofluorescence to determine the percentage of cells possessing surface markers, i.e., the B cell surface immunoglobulin (SIg) marker; the T cell surface antigen Leu-4 (CD3) marker and the monocyte marker MMA. The results are shown in Table 3. Each value represents the mean of three separate buffy coats, i.e., three blood donors. On average one buffy coat consisted of about 55% T cells, 25% monocytes, and 10% B cells.

After passing through a nylon wool column, the purity of T cells increased to about 90%, with only 1% contaminating monocytes and a satisfactory recovery rate of 85%. For isolation of monocytes, it was found that, when compared with the adherent method, Percoll™ gradient centrifugation was more efficient in terms of purity and recovery rate, but was less convenient. Monocyte enriched population prepared by Percoll™ centrifugation contained less than 3% T cells. The small population of B cells in peripheral blood samples and relatively low recovery rate (60%) made it difficult to characterize the behavior of RV in this subpopulation.

Table 3: Characterization of subpopulations of lymphoid cells.

Cell Type	Method	Starting material (%)			Purity (%)			Recovery (%)
		MMA[d]	Leu-4[e]	SIg[f]	MMA	Leu-4	SIg	
Monocyte	Percoll[a]	25	—	—	90	3	1	90
	Adherent[b]	25	—	—	80	7	2	70
T cell	Nylon wool	—	55	—	1	90	3	85
B cell	Rosette[c]	—	—	10	3	5	70	70

a: Percoll: Percoll gradient centrifugation.

b: Adherent: plastic surface adherent method

c: Rosette: AET sheep red blood cell rosetting.

d: MMA: Monocyte surface marker.

e: Leu-4 (CD3): T cell surface marker

f: SIg: B cell surface immunoglobulin

In order to obtain consistent data, one buffy coat was usually used for one set of experiments and the result reported as the mean of the data obtained in three experiments.

3.3.2 Stimulation of PBMC and subsets by optimal doses of mitogens

Polyclonal mitogen activation is one of the most useful systems used to study the mechanisms of activation, growth, and maturation of lymphocytes. However, optimal doses for stimulation with these agents vary, depending on the purity of the preparations, and the exact culture conditions (Weir, 1984). In order to obtain the maximum stimulation of lymphoid cell proliferation in the system used, titration of mitogens was carried out by measurement of [^{3}H]-thymidine uptake by treated cells. Fig. 9A shows the titration of PHA on unfractionated PBMC.

PBMC (2 x 10^6 cells/ml) were stimulated with different concentrations of PHA and pulsed with [^{3}H]-thymidine (14.8 KBq/well) 18 h before harvesting. The CPM in PBMC in 3-day cultures were higher than those obtained in 2-day culture with the same concentration of mitogen. The peak PBMC proliferation response was observed in the presence of 10 µg/ml PHA in both 2 day or 3 day cultures.

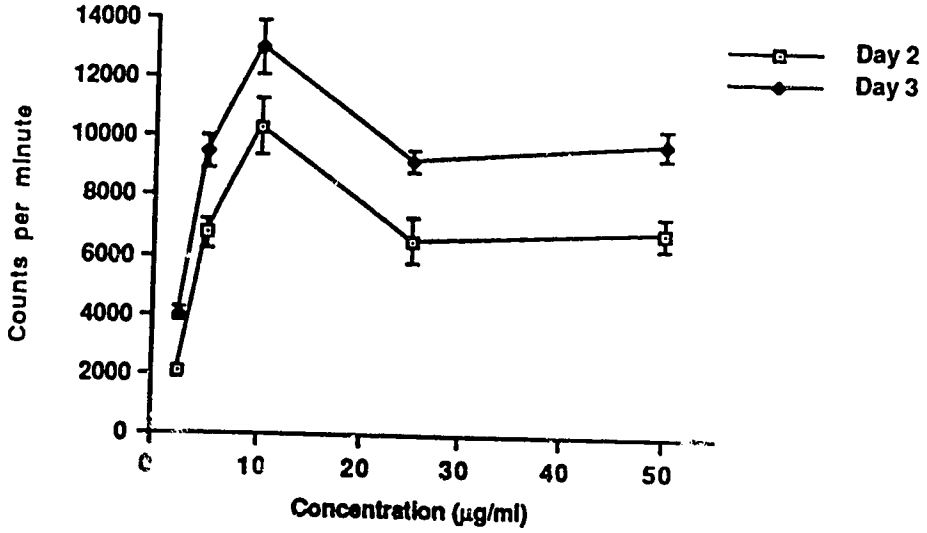
The optimal dose of LPS for monocytes was also determined as shown in Fig. 9B. At 25 µg/ml LPS, monocytes

Figure 9. Mitogen titration. Cells (5 x 10^4 cells/0.1 ml /well) were stimulated with different doses of mitogen and pulsed with 14.8 K' ,/well (0.4 μCi/well) of [3H]-thymidine for the last 18 h before harvesting. Points and bars represent the means ± SD of CPM from six wells.

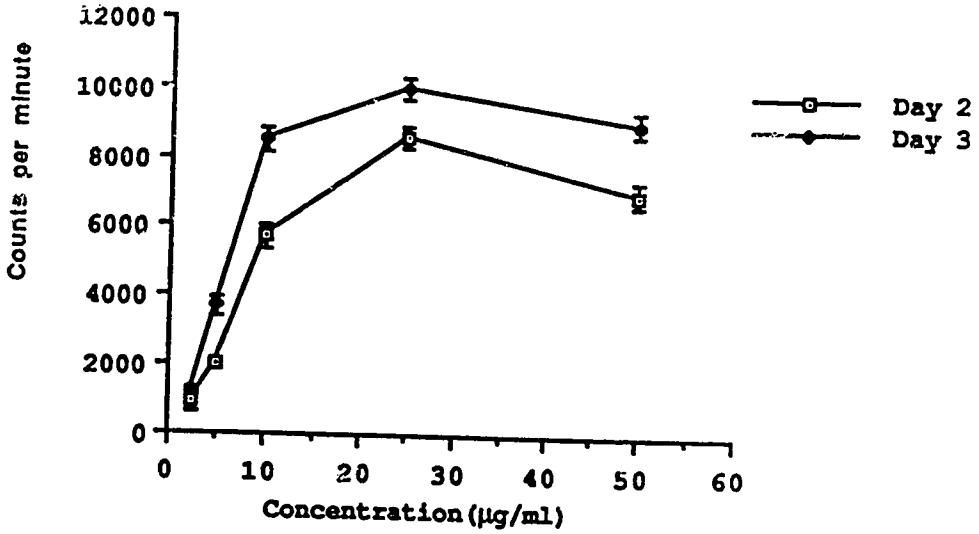
Figure 9A. Optimization of PHA induced responses in PBMC.

Figure 9B. Dose response curve to LPS of monocyte enriched populations.

A


14000
12000
10000
8000
6000
4000
2000
0
Counts per minute
0
10
20
30
40
50
Concentration (µg/ml)
Day 2
Day 3

B


12000
10000
8000
6000
4000
2000
0
Counts per minute
0
10
20
30
40
50
60
Concentration (µg/ml)
Day 2
Day 3

reached maximum proliferation in both 2-day and 3-day cultures. In addition, the optimal dose of PWM for B cells was 10 µg/ml (data not shown). The optimal dose of mitogen was used throughout the following studies.

3.4 One-step replication cycle of rubella virus.

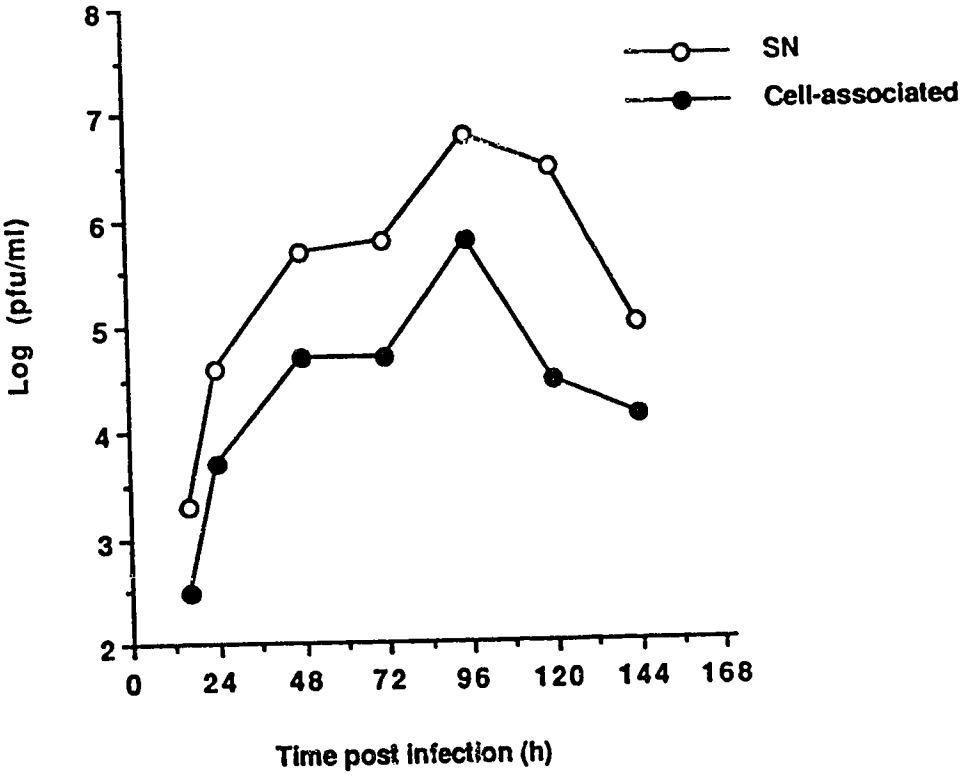
3.4.1 Replication of rubella virus in Vero cell line

Vero cells (1×10^7) were infected at a m.o.i. of 5. Supernatants and infected cells were collected at different times p.i. To obtain cell-associated virus, cells were washed, frozen and thawed, and disrupted by passing through a 18G needle. The titers of infectious virus are shown in Fig. 10.

Vero cells supported productive virus replication. Maximum titers were reached by day 4 p.i. in both supernatant (1×10^7 pfu/ml) and cell-associated virus (5×10^5 pfu/ml) fractions. Overall, the titers in supernatants were higher than those of cell-associated virus.

In subsequent studies, experiments were performed to extend the findings with Vero cells to human peripheral blood mononuclear cells.

Figure 10. One-step growth curve of rubella virus in Vero cell line. Vero cells were infected at a m.o.i. of 5, and incubated in MEM with 5% FCS. Supernatants and cell-associated virus were harvested at each time p.i. and titrated by plaque assay. One-step growth curves from representive experiments are shown.


SN
Cell-associated
Log (pfu/ml)
8
7
6
5
4
3
2
0
24
48
72
96
120
144
168
Time post infection (h)

3.4.2 Replication of rubella virus in PHA-stimulated and unstimulated PBMC

Freshly isolated PBMC (5 x 10^6 cells/ml) were pre-treated with an optimal dose of PHA (10 µg/ml), as determined previously, for about 18-24 h. They were then infected with RV at a m.o.i. of 5. After virus adsorption, PHA was again added into the fresh culture medium. As a control, another group of PBMC received no PHA.

RV released from infected PBMC was titrated by plaque assay on Vero cell monolayers and the results are presented in Fig. 11. In view of the diversity of responses of PBMC obtained from different individuals, the means of results obtained with three buffy coats were used.

In unstimulated PBMC, titers of both supernatant and cell-associated virus were less than 2 x 10^2 pfu/ml on day 2 p.i. and decreased quickly afterwards. Thus, very little, if any replication of RV occurred in resting PBMC.

Maximum virus production was reached by day 2 to day 3 p.i. in PHA treated cultures. Titers of up to 5 x 10^3 pfu/ml were consistently found in supernatants of PHA activated cultures. Titers of cell-associated virus were however, relatively low, only reaching levels up to 2 x 10^2 pfu/ml.

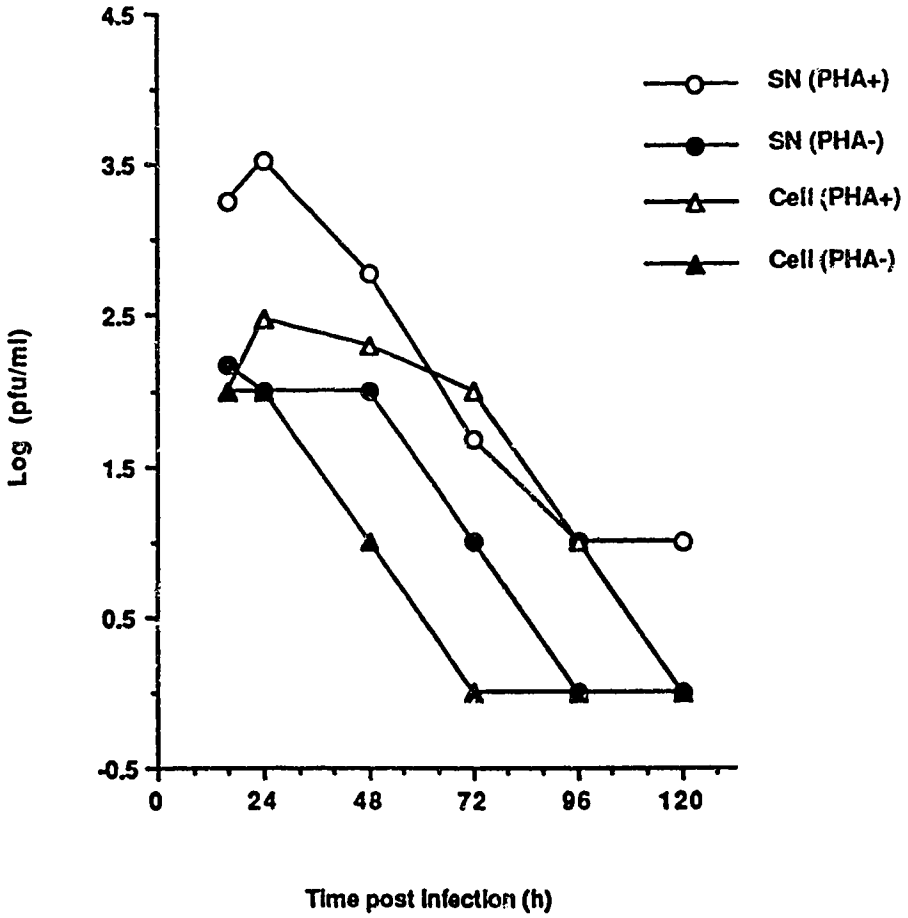
Figure 11. One-step growth curve of rubella virus in PHA stimulated and unstimulated PBMC. PBMC were infected at a m.o.i. of 5. One group of cells was stimulated with an optimal dose of PHA, another group of cells received no PHA. SN and cells were harvested at various times p.i. as indicated and titrated by plaque assay on Vero cell monolayers. The titers shown are means ± SD of pfu/ml from ten buffy coats.

a. SN(PHA+): Supernatant from PHA stimulated PBMC.

b. SN(PHA-): Supernatant from unstimulated PBMC.

c. CELL(PHA+): Cell-associated virus from stimulated PBMC.

d. CELL(PHA-): Cell-associated virus from unstimulated PBMC.


4.5
3.5
2.5
1.5
0.5
-0.5
Log (pfu/ml)
0
24
48
72
96
120
Time post infection (h)
SN (PHA+)
SN (PHA-)
Cell (PHA+)
Cell (PHA-)

Obviously, PHA stimulation was necessary for RV replication in PBMC, although the titers at any time p.i. were lower than those observed in Vero cells.

3.4.3 Replication of rubella virus in monocyte, T cell and B cell enriched populations

Monocyte, T and B cell enriched populations were infected at a m.o.i. of 5. A group of monocytes and T and B cell enriched populations were stimulated with the appropriate mitogens, while another group of these subset cell populations received no mitogen stimulation.

The amount of infectious virus released from the mitogen stimulated cells was averaged and shown in Fig. 12A. Data correlated with Fig. 12A are shown in Table 4. Three phenomena were observed:

Firstly, the amount of infectious virus increased from 16 h p.i. to reach a peak at day 2 or day 3 p.i. Thereafter, virus infectivity decreased and was almost undetectable by day 4 or 5.

Secondly, the titers in monocyte-derived macrophage cultures were higher than those in unfractionated PBMC, but the titers of T and B cell enriched populations were lower than that of the unfractionated population.

Figure 12: One-step growth curve of rubella virus in PBMC and subsets. Cells were infected at a m.o.i. of 5 and subsequently treated with mitogens. SN and cells were harvested at each time p.i. and infected RV was titrated on Vero cell monolayers. The results from three independent experiments were averaged to give the points on the curves.

Figure 12A: RV released into SN of stimulated PBMC and subsets.

Figure 12 B: RV released into SN of unstimulated moncyte, T cell and B cell enriched cultures.

Figure 12C: Titer of cell-associated virus in stimulated monocyte, T and B cell enriched populations.

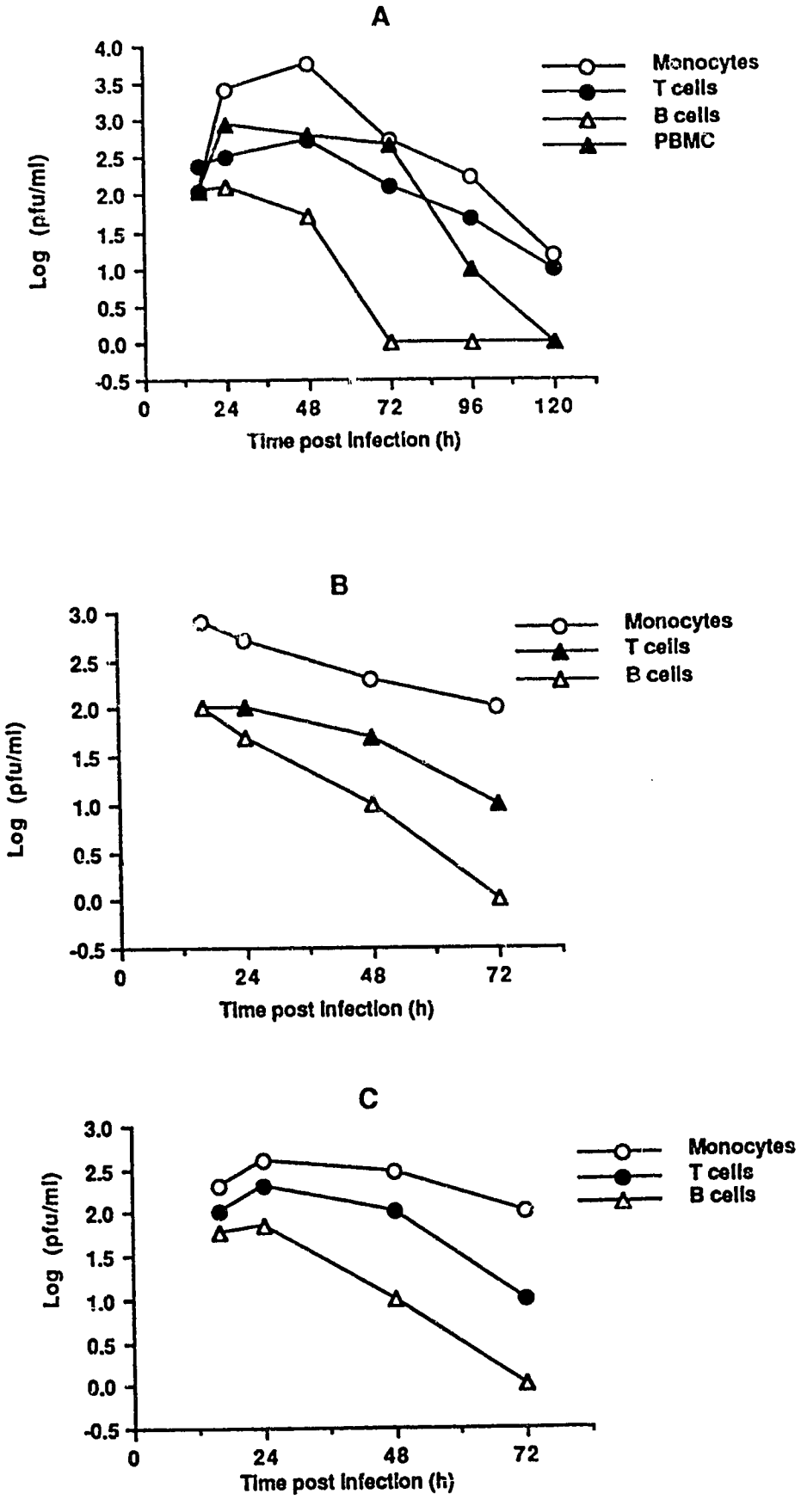

A
Log (pfu/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
24
48
72
96
120
Time post infection (h)
Monocytes
T cells
B cells
PBMC
B
Log (pfu/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
24
48
72
Time post infection (h)
Monocytes
T cells
B cells
C
Log (pfu/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
24
48
72
Time post infection (h)
Monocytes
T cells
B cells

Table 4: Rubella virus production in mitogen stimulated PBMC and subsets. Mean ± SD from three independent experiments correlated with Fig. 12A are shown.

	PBMC		Monocytes		T cells		B cells	
Time (h)	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
16	$1.1x10^2$	$2.3x10^1$	$2.2x10^2$	$2.5x10^1$	$2.5x10^2$	$5.0x10^1$	$1.1x10^2$	$1.2x10^1$
24	$8.6x10^2$	$4.0x10^1$	$2.5x10^3$	$5.0x10^2$	$3.3x10^2$	$1.5x10^1$	$1.3x10^2$	$0.3x10^1$
48	$6.5x10^3$	$5.0x10^2$	$5.7x10^3$	$5.7x10^2$	$5.3x10^2$	$2.6x10^1$	$5.0x10^1$	$0.5x10^1$
72	$4.5x10^1$	$1.5x10^1$	$9.8x10^3$	$2.9x10^2$	$1.3x10^2$	$2.6x10^1$	$1.8x10^1$	$0.3x10^1$
96	$0.6x10^1$	$0.1x10^1$	$1.7x10^2$	$2.6x10^1$	$4.8x10^1$	$3.0x10^1$	0	0
120	$0.2x10^1$	$0.1x10^1$	$1.3x10^1$	$0.3x10^1$	0	0	0	0

Thirdly, with infected B cells, only a small amount of virus was detected at days 2 and 3, and no infectious virus was detected by day 4.

With cell-associated virus preparations (see Fig. 12C) the same phenomena were found, but the titers were lower than the virus released into the supernatant.

For cells with no mitogen stimulation (Fig. 12B), virus titers in the supernatant were not only about one log lower than those detected for mitogen stimulated cells, but also decreased dramatically after day 2 p.i. No virus could be detected in monocyte and T cell enriched cultures by day 4 p.i., nor in B cell enriched culture by day 3 p.i.

The question arose whether the relatively low RV titers found in the supernatants of some infected cultures resulted from the release of input virus, or from productive infection. To answer this question, the following experiments were carried out.

1. Rate of thermal inactivation of RV

High titer stock virus (1×10^7 pfu/ml) was diluted ten fold in RPMI (10% FCS) and incubated alone in a 5% CO_2 atmosphere at 37°C, to mimic the conditions in infected cultures. The diluted stock virus was collected at each time p.i., titrated, and the results are shown in Table 5.

Table 5: Decay rate of diluted stock virus (1:10 dilution). High titered stock virus (0.3 ml) was diluted to 3 ml with RPMI (10% FCS) and incubated alone in a 5% CO_2 atmosphere at 37°C. At each time indicated, a 0.3 ml aliquot of diluted virus was removed and titrated.

Time (h)	Titer (pfu/ml)	Infectivity (%)
0	1.0×10^6	100
2	5.0×10^5	50
4	2.4×10^5	24
12	1.5×10^4	1.5
24	2.4×10^2	0
48	5.0×10^1	0
72	0	0

Approximately 50% of the virus infectivity was lost during the first 2 hours of incubation. After 12 h, almost 98.5% of the virus infectivity had been lost and by 48 h no virus could be detected.

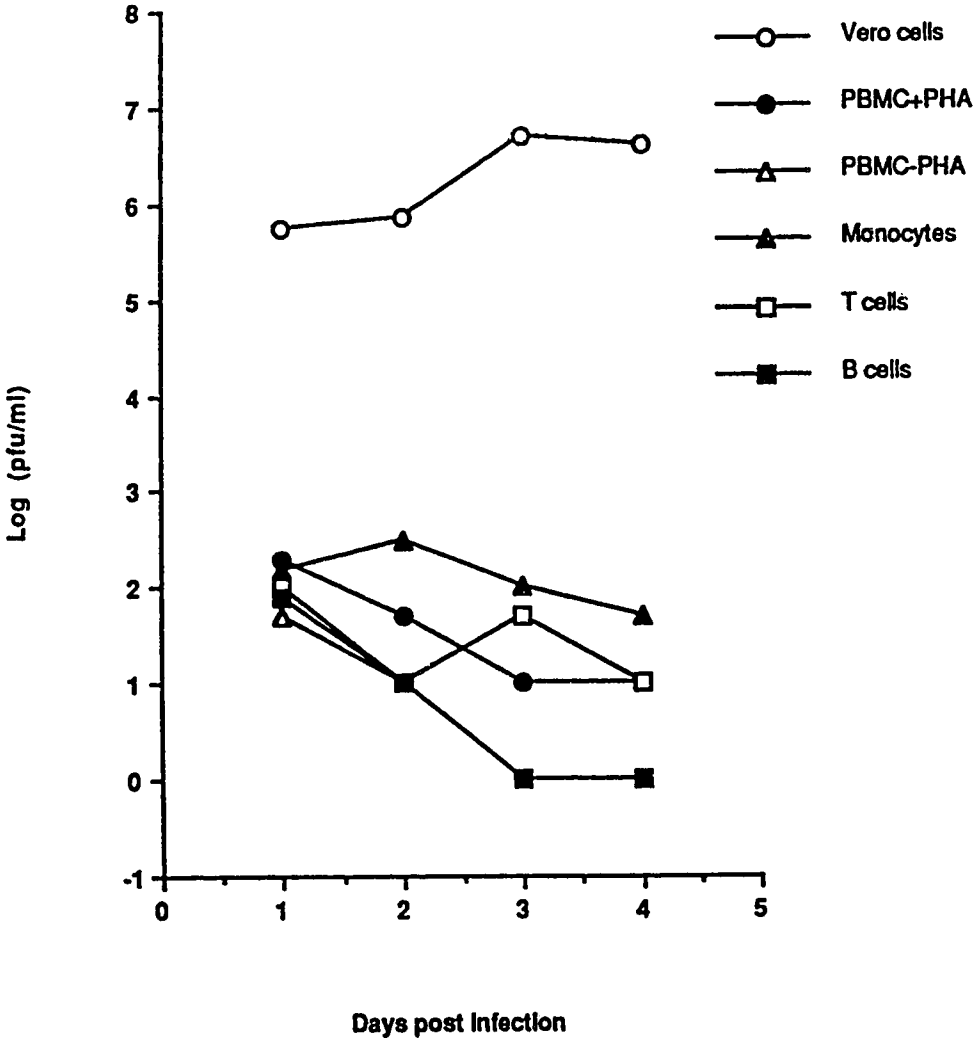
2. RV released into daily culture media

Vero cells, unstimulated PBMC, stimulated PBMC and monocyte, T and B cell enriched populations were infected at a m.o.i. of 5. Fresh medium was added at each day p.i., after harvesting the infected supernatant fluid and washing the cells. Thus it was possible to monitor the virus released on a daily basis, rather than virus accumulation throughout the cultivation period. The result is shown in Fig. 13.

It was noticed that in mitogen stimulated PBMC, as well as enriched monocyte and T cell populations, the virus could be detected at each day p.i. although the amount of virus detected was much lower than that collected from continuous culture medium.

In Vero cells, the amount of virus released at day 3 p.i. was about ten times higher than that at day 2 p.i. and a maximum titer of 1×10^7 pfu/ml was reached. This was almost as high as that obtained in continuous culture medium at 4 days, and was much higher than that obtained from PBMC and monocyte continous cultures.

Figure 13. Rubella virus released into daily culture medium. Vero cells, unstimulated PBMC, or stimulated monocyte, T and B cell enriched populations were infected at a m.o.i. of 5. Fresh medium was added every day p.i. after harvesting the SN and washing the cells. The SN were titrated for infected RV by plague assay.


Log (pfu/ml)
8
7
6
5
4
3
2
1
0
-1
0
1
2
3
4
5
Days post infection
Vero cells
PBMC+PHA
PBMC-PHA
Monocytes
T cells
B cells

However, no daily release of virus could be detected in unstimulated PBMC and stimulated B cell enriched populations.

3. Virus replication after serum neutralization

Unstimulated and stimulated PBMC, monocyte as well as T cell enriched populations were infected at a m.o.i. of 5. Supernatants were harvested and titrated at 24 h p.i. (Table 6). High titer rabbit anti-RV serum (1:10 dilution) was then added to the cells for 1 h at 4°C to neutralize the residual virus adherent on the surface of the cell membrane. The cells were washed vigorously to remove the serum and fresh medium was added. At this point no infectious virus could be detected, but after 24 h of incubation, virus could be detected from stimulated PBMC, monocyte and T cell enriched populations. This virus should represent newly produced virus since the residual cell membrane associated input virus had been neutralized by antiserum. Again, no newly synthesized virus could be detected from unstimulated PBMC.

These results demonstrate that the replication of RV does occur in mitogen-stimulated whole populations of human peripheral blood mononuclear cells as well as in monocyte and T cell enriched populations. No newly synthesized virus could be detected in unstimulated PBMC.

Table 6. Release of virus after antiserum block. Cells were infected at a m.o.i. of 5, supernatants were harvested and titrated at 24 h p.i. Then high titer antiserum was added to the cells for 1 h at 4°C to neutralize any residual virus remaining on the cell membrane. Serum was washed off and fresh medium was added. Supernatants were titrated immediately after neutralization and after a further 24 h incubation at 37°C.

Time (h)	Titer (PFU/ml)			
	PBMC+PHA[a]	PBMC-PHA[b]	Mono+LPS[c]	T cell+PHA[d]
24 h p.i.	100	10	200	0
Neutralization	0	0	0	0
24 h later	40	0	80	20

a: PBMC stimulated by PHA

b: PBMC without PHA stimulation

c: monocyte enriched population stimulated by LPS

d: T cell enriched population stimulated by PHA

3.5 Infectious center assay

An infectious center assay (Table 7) revealed that the number of infected Vero cells increased with time. By day 4 p.i. about 60% of the cells were infected.

The numbers of infected cells in mitogen stimulated PBMC, monocyte and T cell enriched populations also inceased from 16 h p.i. to 72 h p.i. At the peak time of 72 h p.i., the percentage of infected cells was about 7.5% in PBMC; 15% in monocytes; and 5% in T cells.

However, in older lymphoid cell cultures, the percentage of cells suppcrting virus replication decreased. This might be due to the death of the relatively large precentage of lymphoid cells (see Fig. 16).

The increase in the percentage of cells infected provides further evidence that mitogen stimulated PBMC, monocyte and T cell enriched populations could support RV replication to a limited degree *in vitro*.

3.6 Expression of rubella virus antigens in individual infected cells

Firstly, the presence of RV antigen was determined by indirect immunofluorescence (Fig. 14) using high titer rabbit anti-RV serum generated as described previously.

Table 7: Infectious Center Assay. Cells were infected at a m.o.i. of 5 and harvested at the indicated times p.i. After extensive washing, single-cell suspensions were prepared in RPMI (10% FCS) and plated onto Vero cell monolayers. The cell-free supernatant was also analyzed in parallel. The numbers of plaques produced by this cell-free supernatant were subtracted from the experimental data.

Time (h)	Frequency of infectious centers (%)							
	Vero		PBMC		Monocyte		T cell	
	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2
24	20	23	1	2	2	3	1	1
48	31	38	4	4	5	4	2	3
72	55	52	7	8	14	16	4	6
96	60	63	3	4	8	9	3	2

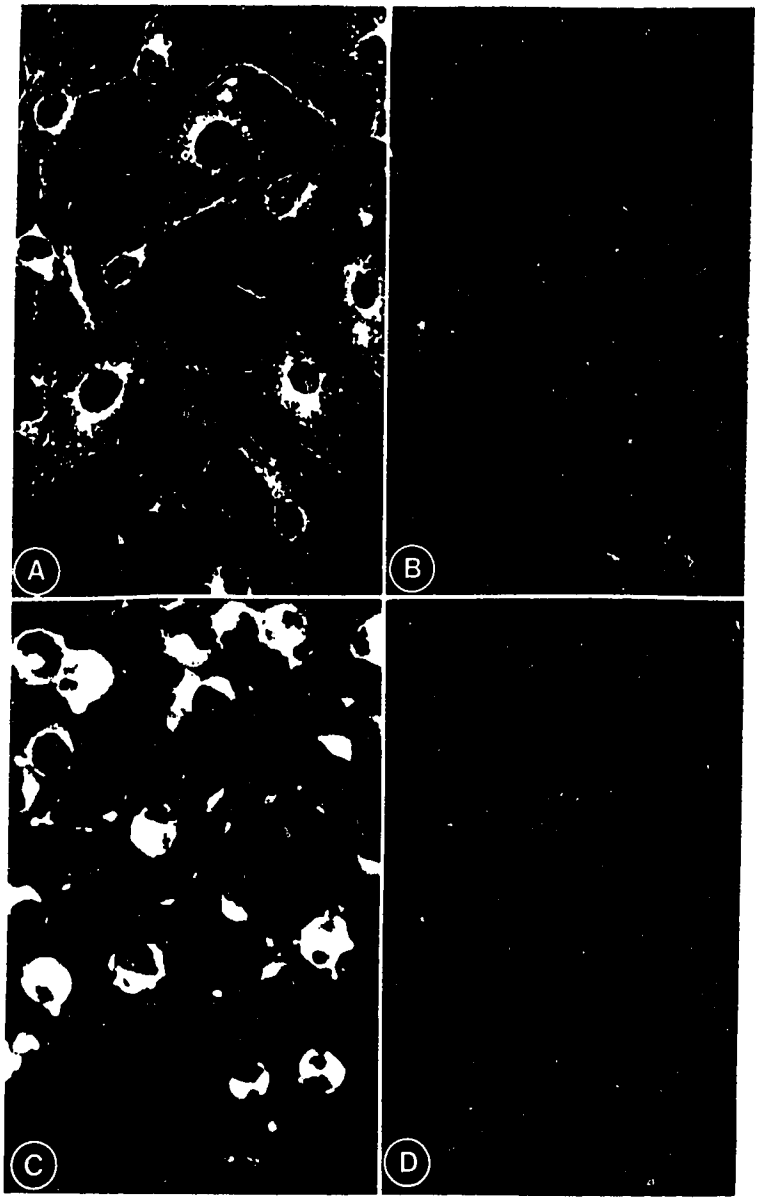
Figure 14. Immunologic localization of rubella virus proteins in infected cells by indirect immunofluorescence. Cells were fixed with cold acetone and immunologically stained with RV specific antiserum as described in Section 2.10.2.

(A) Vero cells 2 days p.i. at a m.o.i. of 5 showing cytoplasmic fluorescence, especially in the perinuclear area (630x).

(B) Mock-infected Vero cells (250x).

(C) Stimulated PBMC 2 days p.i. (630x) showing cytoplasmic fluorescence.

(D) Stimulated mock-infected PBMC (400x).


A
B
C
D

Both infected Vero cells and PBMC (Fig. 14A, C) showed a cytoplasmic fluorescence, especialy in the perinuclear area, whereas no such fluorescence was found in uninfected cells (Fig. 14B, D)

The percentages of antigen positive cells were calculated by counting a minimum of one hundred cells in each preparation. The results are shown in Table 8.

Antigen could be detected at 16 h p.i. and the percentages of antigen positive cells increased over time in all sets of cells. With Vero cells, more than 70% of the cells were positive at 72 h p.i. With PBMC, 17% of stimulated but only 7.5% of unstimulated cells expressed antigen at 72 h p.i. About 20% of monocyte, 12.5% of T cell and 5% of B cell enriched populations were positive at 72 h.

Detection of viral antigen expressed in the B cell enriched population and unstimulated PBMC, (Table 8) indicated that viral specific proteins were synthesized in cells of these populations. However, the fact that very little virus could be detected from the supernatants might be due to (i) the low thermal stability of the proteins or virion, (ii) blockage at either the virus release or assembly stage, or both.

Secondly, a double-labeled fluorescent antibody technique was used to detect the presence of viral antigens in different cell populations of PBMC by the simultaneous

Table 4: Rubella virus production in mitogen stimulated PBMC and subsets. Mean ± SD from three independent experiments correlated with Fig. 12A are shown.

	PBMC		Monocytes		T cells		B cells	
Time (h)	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
16	$1.1x10^2$	$2.3x10^1$	$2.2x10^2$	$2.5x10^1$	$2.5x10^2$	$5.0x10^1$	$1.1x10^2$	$1.2x10^1$
24	$8.6x10^2$	$4.0x10^1$	$2.5x10^3$	$5.0x10^2$	$3.3x10^2$	$1.5x10^1$	$1.3x10^2$	$0.3x10^1$
48	$6.5x10^3$	$5.0x10^2$	$5.7x10^3$	$5.7x10^2$	$5.3x10^2$	$2.6x10^1$	$5.0x10^1$	$0.5x10^1$
72	$4.5x10^1$	$1.5x10^1$	$9.8x10^3$	$2.9x10^2$	$1.3x10^2$	$2.6x10^1$	$1.8x10^1$	$0.3x10^1$
96	$0.6x10^1$	$0.1x10^1$	$1.7x10^2$	$2.6x10^1$	$4.8x10^1$	$3.0x10^1$	0	0
120	$0.2x10^1$	$0.1x10^1$	$1.3x10^1$	$0.3x10^1$	0	0	0	0

staining of cells containing both rubella antigen and surface marker.

The results in Table 9 indicate that among RV antigen positive cells, more than 65% were bearers of monocyte surface marker (MMA) and 15 to 18% were T cell surface marker (Leu-4) bearers. These percentages agree with the previous observation that monocyte or monocyte derived macrophages comprise the main population of cells supporting virus replication (van der Logt et al., 1980).

3.7 Effect of m.o.i. on virus replication

It has been suggested that the m.o.i. could be a significant factor in establishing virus infection in lymphoid cells (Barth and Chantler 1987).

Table 10 shows that at a m.o.i. of 0.1 - 1, the infection was not efficient. When the m.o.i. was increased, the number of cells infected increased. At a m.o.i. of 100, the amount of supernatant and cell-asscociated virus and the number of antigen positive cells increased, but only to a limited extent, when compared with infected Vero cells.

3.8 Effect of rubella virus infection on PBMC and subset blast transformation responses

Freshly isolated PBMC as well as enriched monocyte and T cell populations were infected at a m.o.i. of 5 and stimulated with mitogen. [^{3}H]-thymidine (14.8 KBq/well) was

Table 9: Double-labeling of stimulated PBMC for both cell surface markers and intracellular RV antigns.

Cell	surface marker	percentage of infected cells positive for surface marker[a]		
		Donor 1	Donor 2	Donor 3
PBMC	MMA	66	63	71
	Leu-4	17	18	15

(a) The presence of the surface marker (MMA or Leu-4) and viral antigen in PBMC was determined by simultaneous staining of cells containing both rubella antigen and surface marker at day 2 p.i. The percentage of antigen positive cell bearing surface marker was calculated by changing filters on the fluorescence microscope. [Antigen positive cells /surface marker positive cells]

Table 10: The effect of m.o.i. on virus replication. Cells were infected at different m.o.i. on day 2 p.i. SN and cells were collected and titrated by plaque assay. Infected cells were also analyzed by indirect immunofluorescence with rabbit anti-RV positive serum.

Cell type	MOI	SN Titer	Cell-assoc. Titer	IF positive (%)
PBMC	0.1	<10	<10	1
	1	1×10^2	1×10^1	5
	10	5×10^3	2×10^2	20
	100	8×10^3	5×10^2	29
Monocyte	0.1	<10	<10	2
	1	5×10^2	1×10^1	7
	10	8×10^3	2×10^2	26
	100	3×10^4	1×10^3	40
T cell	0.1	<10	<10	0
	1	1×10^2	5×10^1	2
	10	6×10^2	2×10^1	12
	100	1×10^3	4×10^2	20

added 18 h before harvesting. The results are shown in Fig. 15. When infected PBMC and T cell enriched populations were compared with mock-infected (Fig. 15B, C), a significant decrease in responsiveness to PHA was found by day 3 p.i. A marked decrease in responsiveness to LPS was also found in infected monocyte enriched population at day 3 p.i. (Fig. 15A).

To determine whether the effect was due to any decreased viability of infected cells, Trypan Blue exclusion tests were carried out simultaneously (Fig. 16). The result showed that the numbers of live cells in infected cultures decreased relatively more quickly than those in mock-infected cultures, but the numbers of cells surviving from 3 to 5 days in the two cultures were not significantly diffferent. Therefore, the suppression of PBMC responses to mitogen was not due to a direct cytocidal effect of the virus. In summary, the above data illustrate that RV infection depresses PBMC and subsets responsiveness to mitogens.

In addition, lymphoid cells incubated with virus and washed before stimulation with mitogens gave a reduced response to these mitogens indicating that virus must either attach to or enter those resting cells.

Figure 15. Effect of rubella virus infection on PBMC and sub-population blast transformation responses. Mock-infected or infected cells (5 x 10^4 cells/0.1 ml/well) were pulsed with 14.8 KBq/well (0.4 µCi/well) of [^{3}H]-thymidine for the last 18 h before harvesting. Means ± SD of CPM from six parallel wells are shown.

A. Mock- and infected monocytes enriched population

B. Mock- and infected PBMC

C. Mock- and infected T cell enriched population

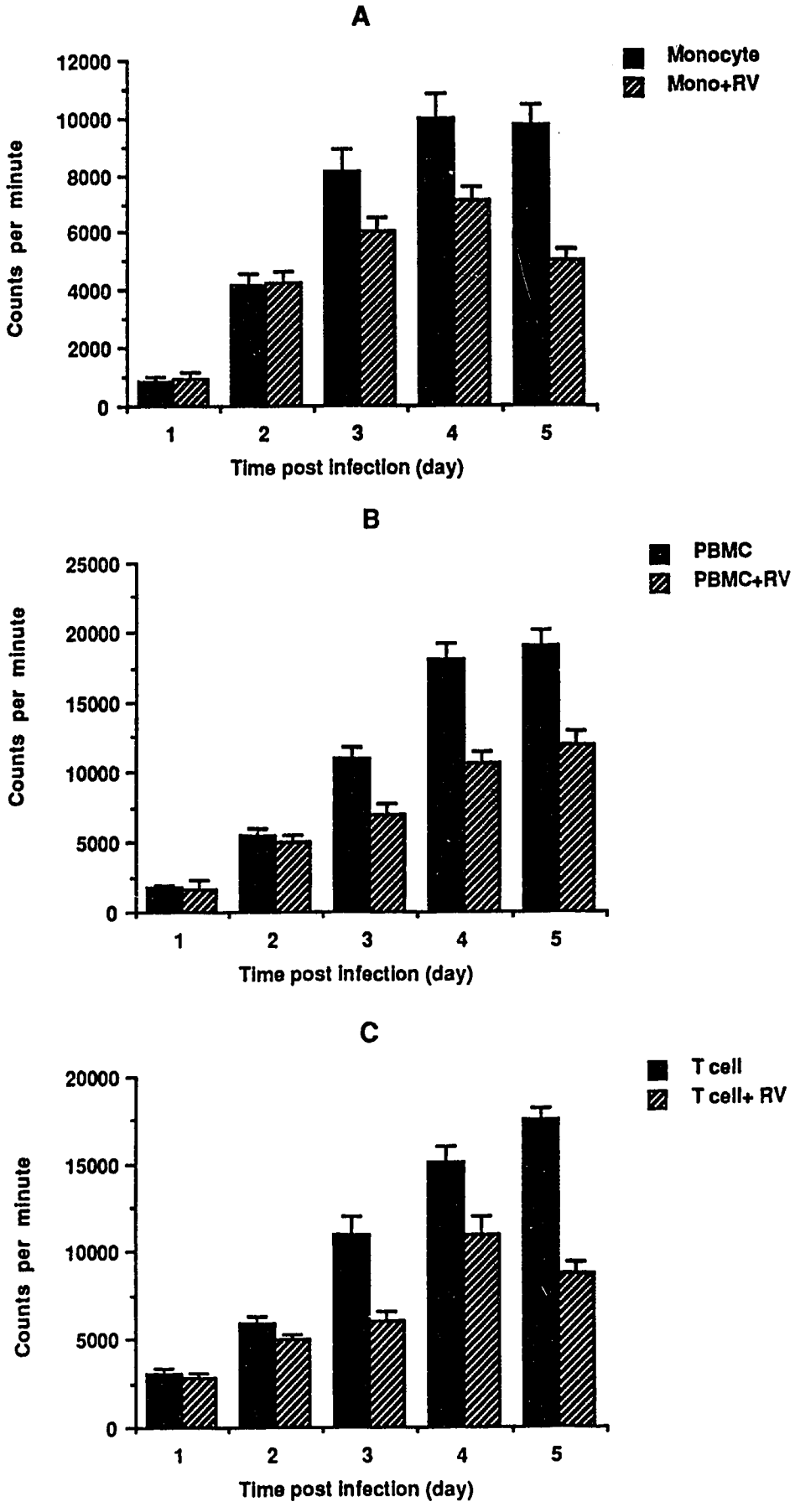
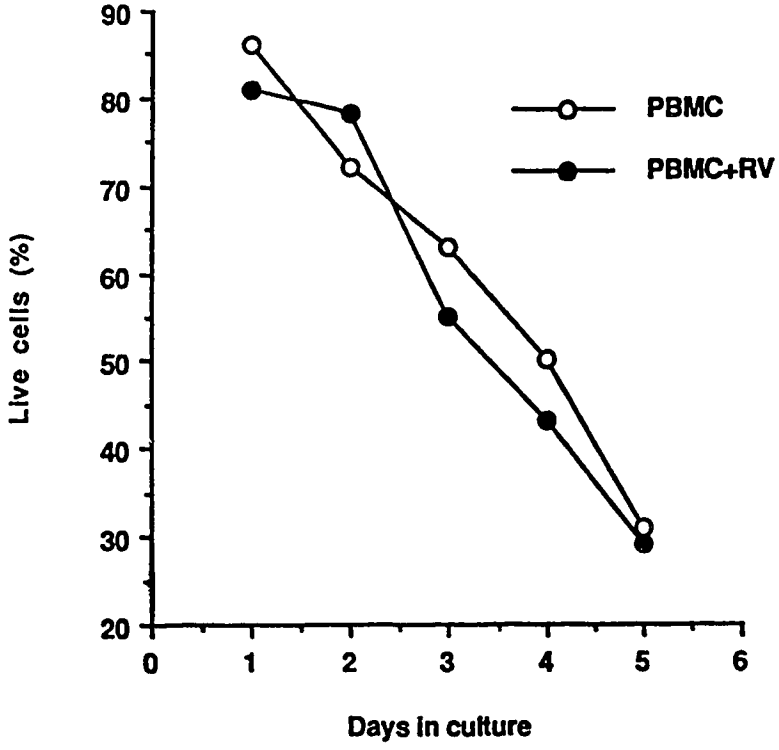

A
Monocyte
Mono+RV
Counts per minute
12000
10000
8000
6000
4000
2000
0
1
2
3
4
5
Time post infection (day)
B
PBMC
PBMC+RV
Counts per minute
25000
20000
15000
10000
5000
0
1
2
3
4
5
Time post infection (day)
C
T cell
T cell+ RV
Counts per minute
20000
15000
10000
5000
0
1
2
3
4
5
Time post infection (day)

Figure 16. Effect of rubella virus infection on the viability of stimulated PBMC. Mock-infected and infected cells were counted by Trypan-blue exclusion at times p.i. indicated. The percentages of live cells were calculated by using the ratio [Number of live cells in culture/Number of total cells in culture]. The results from three independent experiments were averaged to give the points on the curve.


90
80
70
60
50
40
30
20
Live cells (%)
0
1
2
3
4
5
6
Days in culture
PBMC
PBMC+RV

3.9 Synthesis of viral specific proteins in infected cells

To study the rate of viral specific protein synthesis, Western blot experiments were performed with a rabbit anti-RV positive serum using a biotinylated second antibody and streptavidin-peroxidase complexes.

Expression of 62-, 42-, and 35 KD viral proteins was detected in purified virus antigens (Fig. 17B). The C protein migrated further than expected, which might be due to protease-related degradation occuring during the sample preparation (Katow and Sugiura, 1988). Meanwhile, expression of viral proteins in PHA stimulated PBMC could not be detected at 12 h p.i. (Fig. 17C, lane b). Beginning at 16 h p.i. (Fig. 17C, lane c) and at every time point through 48 h p.i. (Fig. 17C, lanes d, e), a relatively stronger signals of E1 and E2 were detected, particularly at 48 h p.i. The C protein was undetectable. None of the viral proteins could be detected in mock-infected cells (Fig. 17C, lane a). The expression of viral proteins in the stimulated monocyte enriched population (Fig. 17A) could also be detected at 16 h p.i. (Fig. 17A, lane a), and the amount of viral proteins synthesized appeared to be similar at each time point (Fig. 17A, lanes b, c). All the signals detected in the monocyte enriched population were stronger than those in unfractionated PBMC.

Figure 17. Western blot analysis of rubella virus specific proteins expressed in stimulated PBMC and monocyte enriched populations. Purified viral antigen and lysates from mock- and infected cells were applied to 10% SDS-PAGE and detected with an rabbit anti-RV serum.

Panel A: viral proteins expressed in stimulated monocyte enriched population.

Lane a: Infected cells, lysed at 16 h p.i.

Lane b: Same as a, 24 h.

Lane c: Same as b, 48 h.

Panel B: Purified RV showed RV specific protein E1, E2 and C.

Panel C: RV protein expressed in stimulated PBMC.

Lane a: mock-infected cells

Lane b: Infected cells, lysed at 12 h p.i.

Lane c: Same as b, 16 h.

Lane d: Same as b, 24 h.

Lane e: Same as b, 48 h.


C
a b c d e
B
A
a b c
E1
E2
C

Radio-labeled cytoplasmic extracts from mock-infected and infected cells were immunoprecipitated with the same rabbit anti-RV serum generated as described previously (see section 2.5). The results are shown in Fig. 18.

Similarly, virus specific proteins were first detected at 16 h p.i. in Vero cells and stimulated PBMC as well as monocyte and T cell enriched populations.

In infected PBMC and monocyte and T cell enriched populations collected after 16 h p.i., the virus specific protein E2 could be clearly seen as two bands (Fig. 18B, C, D). They were designated as E2a and E2b by Oker-Blom et al. (1983).

3.10 Virus specific RNA synthesis

3.10.1 Characterization of cDNA

The plasmid pKTH345 containing a RV cDNA insert was cloned into the *Pst* I site of pBR322 by the dC-dG-tailing procedure (Vidgren et al., 1987). The location of the cDNA insert relative to the C, E2, and E1 genes in the 24S mRNA is shown in Fig. 19. The insert in pKTH345 is derived from the structural genes and hybridizes both with the genomic 40S RNA and the subgenomic 24S mRNA.

Using the published cDNA sequence of the plasmid pKTH345, a computer search of restriction endonuclease sites was carried out. Several restriction sites were found to be

Figure 18. Time-course of viral protein synthesis detected by immunoprecipitation. Mock-infected (M) or RV-infected cells (m.o.i.= 5) were labeled with 1.85 MBq/ml (50 µCi/ml) [^{35}S]-methionine for 16 h and chased with unlabeled medium for 30 min after which cytoplasmic extracts were prepared in NET buffer. The times p.i. that extracts were prepared are indicated at the top of the gel. The position of migration of non-radiolabeled molecular weight markers (KD) on the same gel is indicated on the left-hand margin.

M: Mock-infected cells (M1: 24 h, M2: 48 h, M3: 72 h p.i.).

A: Vero cells at 12, 16, 24, 48 and 72 h p.i.

B: Stimulated T cell enriched population at 16 and 48 h p.i.

C: Stimulated monocyte enriched population at 16 and 48 h p.i.

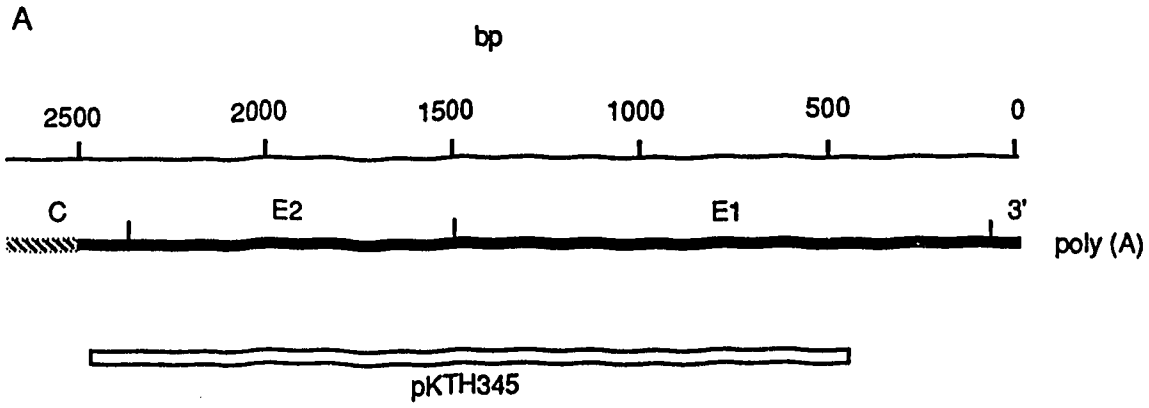
D: Stimulated PBMC at 16 and 48 h p.i.

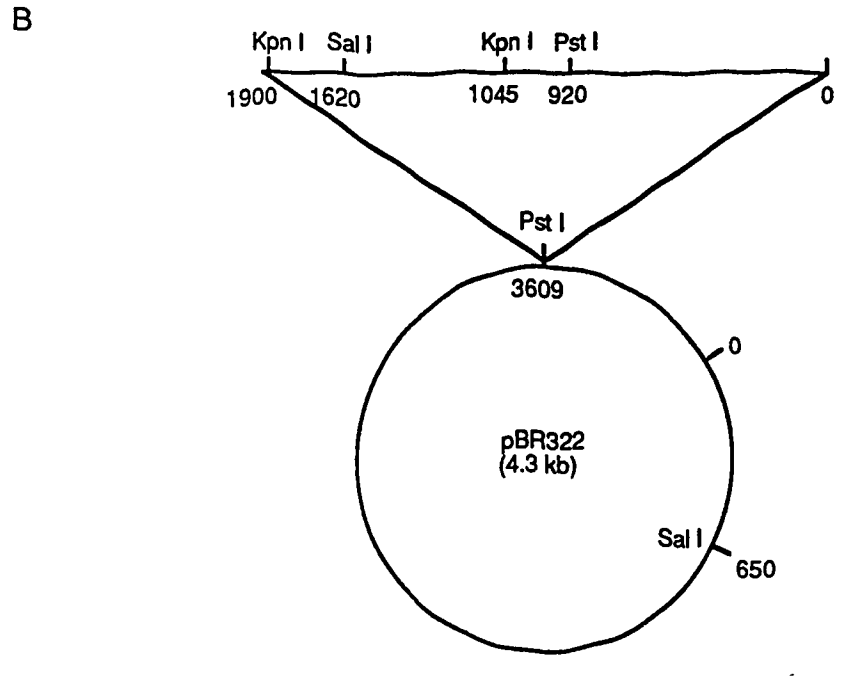

E1
E2a
E2b
C
D
48
16
M1
C
48
16
M1
B
48
16
M1
A
72
16
M3
48
1b
M2
24
12
M1
92
66
45
31
21
14

Figure 19. Cloning strategy for the E1 and E2 genes of rubella virus. cDNA cloned into pKTH345 was obtained as described in *Materials and Methods* using RV 40S RNA as a template.

A The location of the cDNA insert relative to the C, E2 and E1 genes in the 24S mRNA is shown.

B. The construction of pKTH345 is shown.


A
bp
2500
2000
1500
1000
500
0
C
E2
E1
3'
poly (A)
pKTH345


B
Kpn I
Sal I
Kpn I
Pst I
1900
1620
1045
920
0
Pst I
3609
0
pBR322
(4.3 kb)
Sal I
650

usable in restriction map analysis. One of these is the *Pst* I site, which is located close to the middle of the insert. As the cDNA insert was cloned into the plasmid by *Pst* I sites, upon digestion with *Pst* I, two fragments were expected to be released: one about 920 bp and another about 980 bp.

The plasmid was used to transform *E.coli* HB101 and the cells were grown on LB/Tetracycline plates. The antibiotic resistant colonies were picked and amplified. The plasmid DNA was isolated by both small- (mini-prep) and large-scale preparation methods (Maniatis at al., 1982). A portion of the DNA was digested by *Pst* I and analysed on an agarose gel. Fig. 20, lane B, showed that colony contained inserts that had the expected banding pattern of the DNA fragments coding for the partial sequence of E1 (≈920 bp) and the complete sequence of E2 (≈980 bp).

The DNA was further digested with two other restriction endonucleases: those enzymes were *Pst* I, *Kpn* I and *Sal* I. Samples were resolved by 5% PAGE in TBE buffer. The results (see Fig. 21) confirmed that the fragments obtained after digestion corresponded to the estimated theoretical length of the DNA between restriction endonuclease sites. The matching experimental fragments with the estimated theoretical data are shown in Table 11.

Figure 20. Agarose gel electrophoresis of pKTH345 DNA digested with *Pst* I. The pKTH345 insert isolated from a small-scale preparation was digested with *Pst* I overnight at 37°C. Samples were run on 1% agarose in tris-acetate-EDTA (TAE) buffer for 3 h at 60 V. Bands were visualized by ethidium bromide.

Lane A: DNA extracted from antibiotic resistant colony.

Lane B: λ DNA/*Hind* III was used as molecular weight markers (bp).


A
B
4361
2027
564

Figure 21. Polyacrylamide gel electrophoresis of pKTH345 DNA digested with three restriction endonucleases. The pkTH345 insert isolated from a large-scale preparation was digested respectively with three restriction endonucleases for 1 h , at 37°C. Samples were separated on a 5% polyacrylamide gel for 3 h, at 30 mA, buffered by 89 mM Tris-boric acid and 2 mM EDTA, pH 8.0 (TBE).

A. Molecular weight makers (bp): λ DNA digested with *Hind* III and *Eco* RI.

B. pKTH345 digested with *Pst* I.

C. pKTH345 digested with *Kpn* I.

D. pKTH345 digested with *Sal* I.

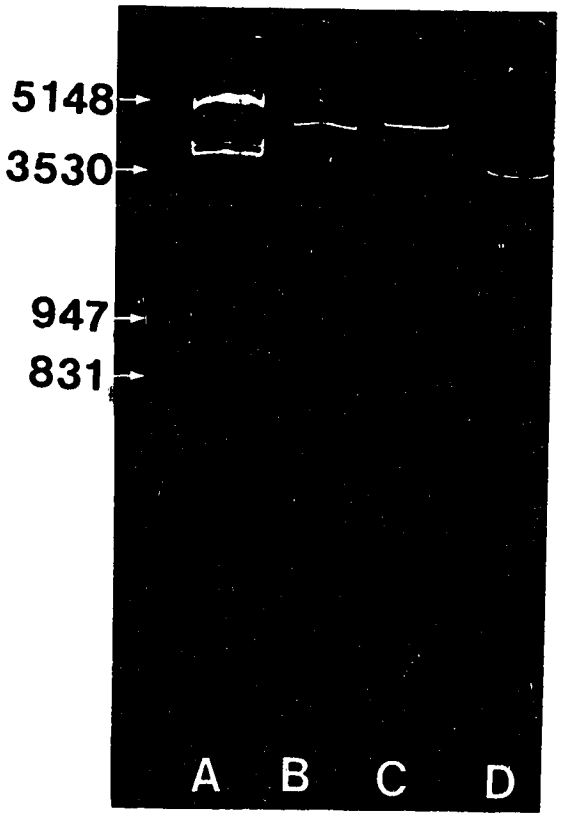

5148
3530
947
831
A
B
C
D

Table 11. Restriction endonuclease map analysis.

Reatriction endonucleases	Theoretical size[a] of fragments (bp)	Experimental size[b] of fragments (bp)
Pst I	4300	4300
	980	930
	920	900
Kpn I	5445	5000
	860	760
Sal I	3200	3500
	3000	3100

(a) The theoretical restricted fragments are derived from the construct shown in Fig. 20.

(b) The size of experimental restricted fragments was calculated from a semi-log plot based on the sizes of *Eco* RI and *Hind* III digested λ DNA fragments and their mobility as standards on the same TBE PAGE.

The above results confirmed that the cDNA had been successfully amplified.

3.10.2 Characterization of extracted RNA

Total cellular RNA was extracted from mock-infected or infected cells and the quality of the RNA was analyzed by formaldehyde gel electrophoresis. The gel pattern (Fig. 22) shows that both 28S and 18S eukaryotic ribosomal RNAs were detectable and the intensity of the 28S band was about twice of that of the 18S band, which suggested minimal RNA degradation during the process of RNA isolation.

3.10.3 Slot blot

Intracellular RNA extracted from mock-infected or infected cultures was denatured in formamide and formaldehyde. After slot blotting onto nitrocellulose, the non-radioactive labelled probes were used to detect the viral specific RNA (see Fig. 23).

The viral RNA was first detected at 12 h p.i. in stimulated and unstimulated PBMC, as well as monocyte and T cell enriched populations. However, the intensity of RNA bands at 12 h p.i. and 24 h p.i. were similar among all cell populations.

Figure 22. Extracted cellular RNA resolved by formaldehyde gel electrophoresis. Extracted RNA (20 µg) and RNA markers were denatured in 6% formaldehyde and 50% formamide at 65°C. Samples were applied to 2.2 M formaldehyde gels in MOPS/EDTA buffer and electrophoresed for 4 h at 100V.

Lane A: RNA Marker: 16S-23S ribosomal RNA from *E.coli*.

Lane B: RNA extracted from mock-infected PBMC.

Lane C: RNA extracted from infected PBMC.

28S and 18S eukaryotic ribosomal RNA's were detected in both B and C.

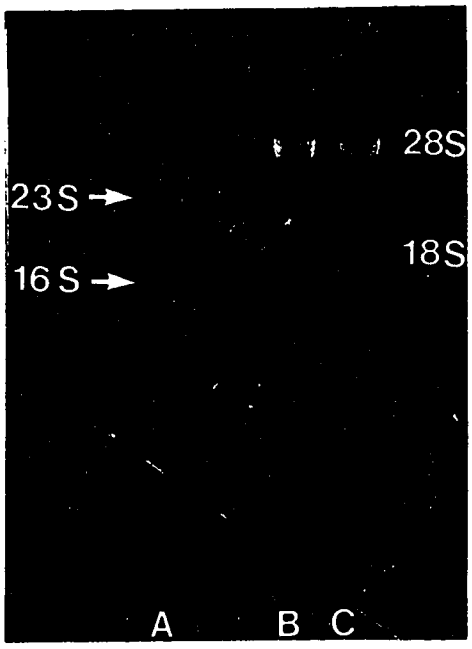

28S
23S
18S
16S
A
B
C

Figure 23: Analysis of viral RNA synthesis by slot blot. Aliquots of total RNA extracted from mock-infected or infected Vero cells (10 µg) and lymphoid cells (20 µg) were denatured in formamide and formaldehyde, slot blotted onto nitrocellulose membranes, and hybridized to the non-radioactive probes. (see section 2.18)

The times p.i. (12 h and 24 h) of the extractions are indicated at the top of the gel.

M: Mock-infected cells.

A: Vero cells.

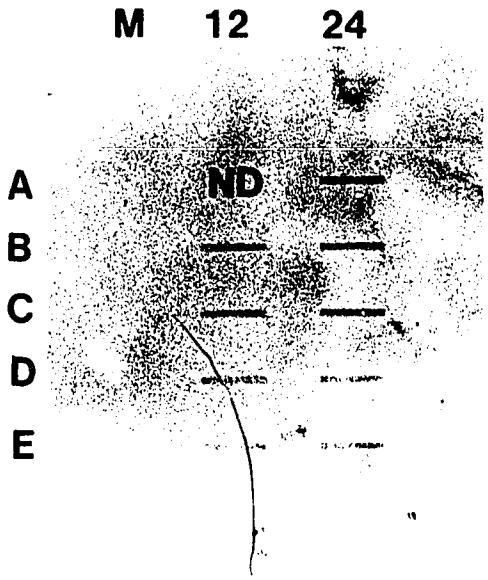
B: Stimulated PBMC.

C: Stimulated monocyte enriched population.

D: unstimulated PBMC.

E: Stimulated T cell enriched population.

*ND = not done


M
12
24
A
B
C
D
E
ND

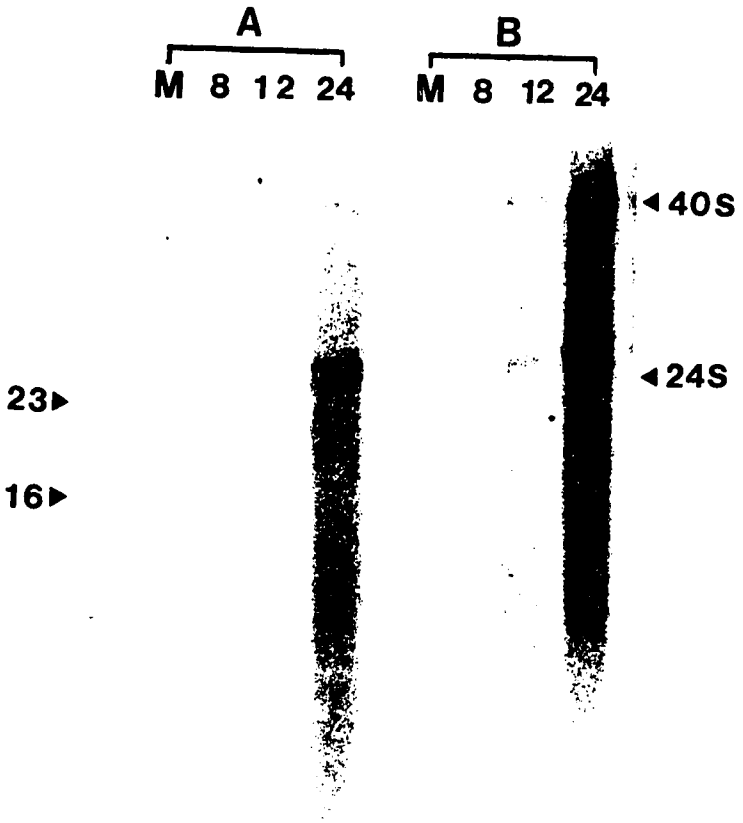
3.10.4 Northern blot

For more specific analysis of the viral RNA species, RNA samples from mock-infected or infected Vero cells (20 µg) and from PBMC (30 µg) were fractionated on formaldehyde gels transfered onto nitrocellulose by Northern blotting and hybridized with [^{32}P]-labelled probes. Autoradiography (Fig. 24) showed the kinetics of viral RNA synthesis in Vero cells and PBMC. Viral genomic (40S) and subgenomic (24S) RNA species were present at each time p.i. tested (12 and 24 h)in both infected Vero cells and PBMC, although the intensity of bands from Vero cells was stronger. Viral specific RNAs smaller than these species could not be detected, which suggested that at least no E1 or E2 related DI particles were produced in the populations of infected cells examined.

Figure 24. Analysis of viral RNA synthesis by Northern blot. Aliquots (20 µg) of total RNA extracted from mock infected or infected Vero cells, and aliquots (30 µg) of total RNA extracted from mock-infected or infected PBMC as well as RNA markers were denatured and resolved on a 2.2 M formaldehyde gel in MOPS/EDTA buffer. Northern transfer and hybridization with the [^{32}P]-labeled probes (see section 2.19) was then performed. The positions of markers *E.coli* 16S-23S ribosomal RNAs are shown in the left hand margin.

Panel A: RNA extracted from mock-infected (M) and infected (8 h, 12 h and 24 h p.i.) PBMC stimulated with PHA.

Panel B: RNA extracted from mock-infected(M) and infected (8 h, 12 h and 24 h p.i.) Vero cells.


A
B
M 8 12 24
M 8 12 24
40S
24S
23
16

4. DISCUSSION

4.1 Basic characteristics of RV replication

It has been reported that production of RV is inefficient and labour intensive (Terry et al., 1989). The difficulty in obtaining sufficient amounts of highly purified virus is obviously a limiting factor to further study of the molecular biology of RV (Oker-Blom, 1984). Therefore, the first goal in this study was to characterize the growth of the virus and to obtain the highest possible titers of stock virus. In this study, characterization of the RV in Vero cells resulted in several observations.

Through the course of observation of RV CPE (Fig. 2), it was found that unlike measles virus-infected cells, the RV infected cell monolayer was always intact as long as the infected cultures were properly maintained. This observation agreed with that of Hemphill et al. (1988). The result suggests that RV has a slow replication cycle, weak cytopathic effect and readily establishes persistent infection in Vero cell culture. These phenomena were also observed in other RV infected cell lines such as RK-13, BHK-21 and LLC-MK_2 (Stanwick and Hallum, 1974).

However, the one-step replication cycle of RV (Fig. 3) showed that the titer of the virus at day 5 p.i. could reach 2.5×10^7 pfu/ml at a m.o.i. of 0.1 in the presence of high concentration of FCS (5%). The result revealed that virus

replication was efficient in Vero cells under optimal culture conditions.

Moreover, it was found that RV infection was more effective in subconfluent cell monolayer (Table 2). Accumulated evidence suggests that the efficiency of RV infection depends on a cellular receptor or factor that is present in abundance in metabolically active cells (Hemphill, et al., 1988). Thus, the more efficient RV infection observed in subconfluent cell monolayers might be due to the more active metabolic stage of the cells.

It was noticed that the RV is relatively fragile. When virus was precipitated with 30% (w/w) sucrose cushion according to a previously published purification method (Oker-Blom, 1984), the yield was dramatically decreased. The modified method using a 20% to 60% sucrose step gradient instead of the precipitation procedure offers the obvious advantage of preserving the virus.

Purified RV analyzed by SDS-PAGE (Fig. 5) had the typical RV structural protein patterns: E1 (62 KD); E2 (48 KD) and C, (35 KD), as described in the literature (Oker-Blom, 1984; Toivonen et al., 1983). EIA (Fig. 4) with purified RV and known RV positive serum showed that the purified virus was antigenic to RV antibody which provided evidence that this strain of RV, under the experimental conditions of this

study, expressed the typical characteristics of wild type rubella virus.

Immunofluoresence studies (Fig. 14) revealed that the expression of viral antigen was located in the cytoplasmic area. It was previously reported that the envelope antigens of togaviruses were transferred through the intracellular membranes during the maturation of these glycoproteins. The earliest step in this maturation occurs in the rough endoplasmic reticulum and the more mature forms of the polypeptides are found in the smooth membranes (von Bonsdorff and Vaheri, 1969). Although this sequential maturation has not been clearly demonstrated for rubella virus (Tuokko et al., 1984), it is reasonable to assume that the same is also true for this virus. Recently, Hobman et al. (1988) found that translocation of RV E1 glycoprotein into the endoplasmic reticulum is mediated by a signal peptide contained within the 69 carboxyl-terminal residues of E2.

Northern blot analysis (Fig. 24) showed that in the stock RV infected cells, both 40S genomic RNA and 24S subgenomic RNA species but not smaller sizes could be detected. Since analysis of intracellular virus-specific RNA species had been used as a sensitive assay for the presence of DI particles in virus stocks (Meinkoth and Kennedy, 1980; Weiss et al., 1980), these results demonstrated that the RV stock used in this study was free of DI particles. It was noted previously that the generation of DI particles by RV was no more rapid

than that other viruses, although RV readily generates DI particles under conditions in which DI RNAs were generated by other RNA viruses (Frey and Hemphill, 1988). Therefore, it was suggested that the establishment of persistence was not necessarily due to the ability of RV to generate DI particles.

4.2 Replication of RV in PBMC and subsets

It has been the experience of many investigators that infection of the lymphoid cells is a prerequisite for abrogation of the host immune function (Wainberg and Mills, 1985). Thus, elucidation of the mechanism of replication of RV in human lymphoid cells is valuable in viral pathogenesis and persistent infection research.

The results of this study are interpreted to indicate that RV replication in PBMC and subsets was subject to three kinds of restrictions.

4.2.1 Mitogenic stimulation of cells is a restrictive factor in RV productive infection.

In the study of the one-step growth curve of RV in PBMC and subsets (Fig. 11, Fig. 12A, B and Table 4), the results indicated that the amount of virus released from mitogen stimulated PBMC, monocytes and T cell enriched populations increased as time elapsed, whereas the amount of virus released from those populations without PHA stimulation were

decreased. Moreover, in unstimulated PBMC, no virus could be detected when culture medium was changed every day (Fig. 13), and no newly synthesized virus could be detected after serum neutralization (Table 6). Taking into account the decay rate of stock virus in medium alone (Table 5), it could be concluded that RV could not cause productive infection in unstimulated PBMC and subpopulations but could do so in mitogen stimuated PBMC, monocyte and T cell enriched populations although the amount of virus released was relatively low (Fig. 11, Fig. 12A, B and Table 4). It has been suggested that prestimulation of PBMC was essential for the production of a high yield of virus (Chantler and Tingle, 1980). However, under the experimental conditions of this study, it was found that whether freshly isolated PBMC were infected with RV immediately before being treated with PHA or treated with PHA for 1, 2 or 3 days prior to the infection, there was not much difference in virus titers (data not shown). A possible explanation is that when small resting lymphoid cells are stimulated by mitogen, the aggregation of the transformed blast cells may not be a suitable situation for virus adsorption and penetration (Dr. Aimo Salmi, personal communication).

Whether the failure of resting PBMC to produce infectious virus was simply because the cells lack the ability to be infected by virus, e.g., lack a RV receptor, or reflected a molecular deficit which was altered upon PBMC activation is

not known. Lymphoid cells incubated with virus and washed before stimulation with mitogens gave a reduced response to these mitogens (Fig. 15) indicating that virus must either attach to or enter resting cells. Similar phenomenona were observed by Knight and Najera (1969). It was likely that resting PBMC could be infected by the virus but required some molecular change upon "activation" to become permissive for the virus replication and release (Bloom et al., 1977).

The presence of virus RNA species in resting PBMC detected by slot blot (Fig. 24) and the presence of RV antigen in this population detected by immunofluoresence (Table 8) indicated that the abortive infection block was at the virus maturation or release stage rather than at the viral macromolecular transcription or translation level.

4.2.2 The type of mitogen stimulated immune cell is another restrictive factor in RV productive infection.

The immunofluorescence results (Table 8) indicated that at 72 h p.i., approximately 17% of the PBMC, 24% of the monocyte and 12.5% of the T and 5% of the B cell enriched populations stimulated by mitogen expressed RV antigens. These cells therefore harboured the virus and might support the productive infection of the virus. Studies of the virus released from mitogen stimulated PBMC and subsets (Fig. 12A and Table 4) revealed that the monocytes generated a higher

titer of virus than T and B cell enriched populations. The infectious center assay (Table 7) also revealed that by 72 h p.i., the percentage of cells supporting RV replication was highest in the monocyte enriched population (15.5%). Among infected cells, more than 65% carried the monocyte surface marker and 15 to 18% carried the T cell marker (Table 9). The remaining 10 to 20% of the RV antigen positive cells may belonged to B cells, NK cells or cther cell populations (Salonen, Ilonen and Salmi, 1988).

These data unequivocally demonstrated that the monocyte enriched population was the principal host cell for virus replication. This was also observed by van der Logt et al. (1980). In addition, it was found that RV antigens were expressed more frequently on monocytes than the T and B cells *in vivo*, as detected by flow cytometry (O'Shea et al., 1988). In measles virus infected unstimulated PBMC, it was also found that virus production occured preferentially in monocytes (Salonen, Ilonen and Salmi, 1988). The monocyte enriched population played a central role in virus infection, which is not surprising as these cells are phagocytic and important for antigen presentation, induction, regulation and amplification of both humoral and cell mediated immune responses (Morahan et al., 1985). A reasonable explanation is that monocytes, when cultivated *in vitro*, rapidly adopted the characteristics of macrophages (Bennett and Cohn, 1966), which may be more susceptible to viruses. The defects and

changes in the cells of the mononuclear phagocyte system can severely impaire the interactions among monocytes, T cells and B cells, and result in immune abnormalities (Edelman and Zolla-Pazner, 1989)

In addition, the selectivity of RV for lymphocytes was obvious. In the studies of the one-step replication cycle (Table 4), at day 3 p.i., the yield of infectious virus in stimulated T cell enriched populations was low (5.3×10^2 pfu/ml). However, further experiments revealed that released progeny virus could be detected in T cell enriched population when culture medium was replaced on a daily basis (Fig. 13). The newly synthesized progeny virus could be detected in the T cell enriched cultures that had been treated with antiserum after infection to neutralize residual virus which might stick on the cell membrane during the course of adsorption (Table 6). Moreover, three viral structural proteins (E1, E2 and C) were detected by immunoprecipitation (Fig. 18) in stimulated T cell enriched populations. Thus, it may be concluded that RV infection is productive to a limited degree in the T cell enriched population.

Infectious virus detected in B cell enriched population at day 3 p.i. was extremely low (5×10^1 pfu/ml), and no virus could be detected when medium was changed every day (Fig. 13). Low level infection possible due to comtaminating monocytes. Taking into account the kinetics of decay of the virus in culture medium alone (Table 5), it could be also

assumed that the infectious particles detected in the B cell enriched population were most likely remnants of the inoculum virus rather than progeny virus. Failure to recover infectious RV in stimulated B cell culture was also observed by van der Logt's group (1980).

The selectivity of RV productive infection in T cells but not in the B cell enriched population is not a unique phenomenon. Similar obsevations have been made for herpes simplex and mumps viruses (Kirchner et al., 1977; Flerscher and Kreth, 1982), Epstein-Barr and human cytomegalovirus (HCMV) (Menezes et al., 1976; Olding et al., 1975). On the other hand, some viruses, e.g., measles virus, can replicate in both activated T and B cells (Sullivan et al., 1975).

Based on immunofluorescence studies (Table 8), about 5% of the B cell enriched population expressed RV antigens. Therefore, it could be assumed that the virus did start the *de novo* synthesis and the envelope antigens were transferred through the intracellular membranes, but blocked in the process of assembly or release stage. The non-permissiveness of B cells or resting PBMC for VSV was also reported previously (Bloom et al., 1977). It has been suggested that the defect lies not at the level of display of receptor on the cells which were infected by virus, but at the level of permitting a complete replicative cycle.

It should be pointed out that Barth and Chantler (1987) found that RV (HPV77/DE5) could replicate in not only CD^{4+} and CD^{8+} T cells in the prescence of PHA and IL-2, but also in B cells stimulated by anti-Ig and B cell growth factor (BCGF) (Maizel et. al., 1983) with high virus yields. They suggested that the previous reports failing to detect infectious virus in B cells (Chantler and Tingle, 1980) and in both T and B cells (van der Logt et al., 1980) were attributed to the lack of pre-stimulation with anti-Ig and BCGF or PHA and IL-2. As already discussed by Rinaldo et al. (1978), the failure of human mononuclear cells to allow replication of virus or semi-permissiveness (e.g. titer of 10^3 pfu/ml) might be caused by a limited survival time of cells in culture. The availability of IL-2 (Smith et al., 1979) might help overcome this problem, at least for T cells. IL-2 may have a role in the initiation of a persistent infection and stimulating proliferation of lymphocytes carrying virus, and consequently promote virus maturation. For example, T cell cultures grown with IL-2 support a limited replication of HCMV between day 11 and 13 p.i., whereas no progeny virus was found in culture without the addition of IL-2 (Braun and Reiser, 1986).

On the other hand, the different virus strain used should be taken into consideration, since a great variability in virus-lymphocyte interactions between measles virus strains has been reported (Ilonen et al., 1988), and a difference in the

mechanism of viral inhibition of lymphocyte proliferation *in vitro* between the strains was recently reported (Ilonen and Salmi, 1989).

From the observations of this study, it was concluded that RV can productively infect stimulated PBMC, monocyte-derived macrophages and T cell enriched populations. In the B cell enriched population, the productive infection may depend on the virus strain used and the condition of stimulation. When compared with the Vero cell line, the PBMC and subsets supported virus replication only to a limited extent. Absence of detectable productive infection in B cells might indicate that these cells served as a long-term harbour for the virus.

4.2.3 The length of immune cell survival *in vitro* is a restrictive factor in RV productive infection

In RV infected Vero cells and PHA stimulated PBMC, both genomic and subgenomic RNA species were first detectable at 12 h p.i. (Fig. 24). Virus structural proteins were first detectable at 16 h p.i. in Vero cells, mitogen stimulated PBMC, monocyte and T cell enriched populations (Fig. 18). The time at which RV RNA and protein synthesis were first detectable agreed with the observation of Hamphill et al. (1988).

In the case of PBMC, the time course of protein synthesis observed coincided with that reported by Chantler and Tingle

(1980) who suggested that structural protein synthesis commences between 16 and 24 h p.i. Immunofluorescence in lymphoid cells was seen at 16 h p.i., and the percentage increased as time elapsed. However, even at the peak time of 72 h, only 17% PBMC, 24% monocyte, and 12.5% T cells expressed virus antigens (Table 8). Thus, only a small percentage of lymphoid cell was involved in RV persistent infection. Similar results were observed in the infectious center assay (Table 7).

It was noted that the sequencial rates of synthesis of RV RNA and protein in Vero cells were similar to those in lymphoid cells. With the same number of cells, and under similar culture conditions, virus yields could reach 2×10^7 pfu/ml in Vero cells at day 4 p.i., whereas only 2×10^2 pfu/ml virus could be detected in the monocyte derived macrophage population by day 4 p.i. (Table 4), the principal type of cells involved in viral infection of lymphoid cells (van der Logt et al., 1980). In addition, an examination of the effect of RV infection on the viability of PBMC (Fig. 16) indicated that in both mock-infected and infected cultures, the percentage of living cells decreased quickly as time elapsed. By day 4 p.i., only 35 to 30% living cells were left in the cultures. This low percentage of living cells might be the limiting factor in the virus replication. It can be concluded that the time of lymphoid cell survival *in*

vitro is likely to be a restrictive factor in RV productive infe · ion in freshly isolated lymphoid cells.

This restriction has been also emphasized in the failure of replication of HCMV in PBMC (Rinaldo et al., 1978; Braun and Reiser, 1986).

4.3 The effect of RV replication on immune cell function

The mechanisms whereby viruses may interfere with the host immune system are probably as varied as the viral agents themselves. One of many suggested mechanisms is viral infection of immunocompetent cells, with alteration of the cellular function, leading to varying extents of immunosuppression in the host. Some viruses such as human immunodificiency virus (HIV) infect and kill cells, Other viruses can simultaneously interrupt several facets of immune responsiveness (Wainberg and Mills, 1985).

A transient or prolonged immunosuppression is a well-established feature of RV infection or vaccination (Morag et. al., 1975), but the mechanisms are not clear. Various effects on PBMC function have been observed as a result of RV infection *in vitro*. For example, polyclonal B cell activation during acute rubella (Hyypia et al., 1984; 1985) clearly demonstrates that the functional activity of mononuclear cells is disrupted. Moreover, it has been observed that RV decreases the blastogenic response to PHA in

lymphocytes from patients with congenital or postnatally acquired rubella or after rubella vaccination (Buimovici-Klein and Cooper, 1979). Our studies revealed that RV infection could depress PBMC and subset responsiveness to mitogens *in vitro* (Fig. 15). The phenomena were also observed by Maller and Soren (1977). The mechanism by which RV depressed the PHA response was not due to direct killing of the cells as RV is not cytocidal (Fig. 16). A similar result was also observed by Buimovici-Klein and Cooper (1979). The depressed response could be due to indirect infection of macrophages, thereby diminishing the enhancing effect of macrophages on the PHA response of lymphocytes (Soontiens and van der Veen, 1973). The diminished responsiveness of RV infected PBMC and its subsets to mitogens means that the virus can impair the function of the immune system to recognize pathogens as foreign and to react against them (Wainberg and Mills 1985). This suppressed immune response may favor the establishment of rubella virus persistence.

5. CONCLUSIONS

The replication of rubella virus in human peripheral mononuclear cells has been studied.

In the first part of the study, the Therien strain of RV has been characterized using a Vero cell line. The results suggested that under the experimental conditions used, this strain expressed the typical characteristics of wild type RV as described in the literature.

In the second part of the study, three types of restriction on the replication of RV in PBMC and subsets were observed. First, it was shown that mitogen stimulation was required for RV productive infection. Resting PBMC can be infected but require some molecular change associated with mitogen stimulation in order to permit maturation of infectious virus. Failure to produce progeny virus was due to blockage at the virus assembly or release stage.

The second restriction was related to the nature of the virus producing cells. Stimulated monocyte-derived macrophages were the principal cells supporting virus production. The T cell enriched population supported virus productive infection to a limited degree. However, RV could not complete the full replication cycle in a stimulated B cell enriched population, and the infection was aborted at the virus maturation stage.

In addition, only a small percentage of PBMC and subsets were involved in RV persistent infection, and the yield of virus in cells of lymphoid system was lower than that in Vero cells.

It was found that RV had the same sequential rate of macromolecular synthesis in Vero cells as in PBMC and subsets, but the efficiency of replication in each was different. Infected Vero cells could survive longer than lymphoid cells *in vitro*, and thus might support better virus replication. Therefore, the length of immune cell survival *in vitro* is likely to be a restrictive factor in RV productive infection in PBMC.

Finally, the effect of RV replication on those immune cell function was studied. The results indicated that virus infection could suppress the responsiveness of PBMC and subsets to mitogens. This alterated immune cell function might be as a cofactor in the immune disturbances in RV infected patients. Therefore, the replication of RV in cells of lymphoid system play an important role in the pathogenesis of persistent infection. Further endeavours to study the relationship between RV and PBMC, and to elucidate the mechanism(s) of virus-mediated immune suppression and persistent infection are required.

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