INFECTION AND IMMUNITY, June 1981, p. 1274-1280
0019-9567/81/061274-07$02.00/0

Vol. 32, No. 3

Rubella-Associated Arthritis: Rescue of Rubella Virus from Peripheral Blood Lymphocytes Two Years Postvaccination

J. K. CHANTLER,[1]* D. K. FORD,[2] AND A. J. TINGLE[3]

Division of Medical Microbiology[1] and Departments of Medicine[2] and Pediatrics,[3] University of British Columbia, Vancouver, British Columbia, Canada

Rubella virus was isolated from the peripheral lymphocytes of a patient with rubella-associated arthritis 2 years after rubella vaccination. The rescue of the virus was carried out by stimulating the lymphocytes with mitogens for several days in cultures and then cocultivating them with RK13 cells at 35°C. Rubella virus was detected by a variety of techniques, including electron microscopy, indirect immunofluorescence, polyacrylamide gel analysis of labeled viral proteins, and microfocus assay for infectious virus particles. Assessment of the immune status of the patient did not indicate any impairment of immune function associated with long-term persistence of the virus.

Acute polyarticular arthralgia constitutes one of the most frequently observed adverse reactions following both natural rubella infection and immunization with live, attenuated rubella vaccine (3, 19). Joint manifestations occur most frequently in adolescents and adults and are usually transient and without late sequelae. However, recurrent episodes of arthritis have been observed several years after rubella infection (8), and in two cases, the symptoms have been reported to progress to rheumatoid arthritis (6, 11).

The pathogenesis of rubella-associated joint manifestations has remained obscure. It has been suggested that the clinical pattern of repeated flare-ups of disease activity followed by periods of quiescence represents the periodic reactivation of a persistent rubella infection. This possibility has been supported by the isolation of rubella virus (RV) from joint aspirates during the initial acute episode of arthritis (7, 17) and during recurrent episodes of arthralgia for several months after vaccination with the HPV-77 DK/12 strain of rubella vaccine (12). Virus persistence may occur in a variety of cell types within the synovial tissue itself or at some other site in the body, the most likely alternative involving cells of the lymphoreticular system. RV is known to replicate in human peripheral blood lymphocytes (PBL) in vitro (2a) and has been shown to infect circulating lymphocytes at least transiently after natural infection or vaccination in vivo (1, 2a). In addition, RV persistence is known to occur for prolonged periods in the PBL of infants with congenital rubella syndrome (15) and in patients with rubella panencephalitis, a delayed-onset manifestation of congenital rubella (18).

This ability of RV to replicate and persist in human lymphocyte populations under various conditions encouraged us to search for RV in the PBL of individuals undergoing recurrent episodes of polyarticular arthritis which followed immunization with the HPV-77 DE/5 strain of rubella vaccine. In addition to virus rescue, the immune status of the patient, including levels of antibody to RV, and lymphoproliferative responses to mitogens and RV antigen were assessed. In this paper, we report the successful isolation, 27 months after rubella immunization, of RV from a 26-year-old female with recurrent arthralgia. There was no evidence of dysfunction of either the humoral or the cell-mediated immune response.

MATERIALS AND METHODS

Case history. A 26-year-old laboratory technician was immunized on 28 December 1976 with the HPV-77 DE/5 strain of rubella vaccine. She had been in good health previously, had no past history of arthritis or other joint problems, and had a preimmunization rubella hemagglutination inhibition titer of <1:8. Fifteen days after immunization, she developed a sore throat, low-grade fever, and fatigue, followed on the subsequent day by the development of pain and swelling in the right ankle and knee and pain in the wrists and fingers. The swelling of the knees and ankles persisted for 3 to 4 days, and the arthralgias subsided over the next week. A second similar attack occurred 29 days after immunization, and over the next 3 months, she continued to have recurrent episodes of mild arthritis involving the knees, ankles, wrists, fingers, and feet. Over the subsequent 6 months, she had intermittent episodes of arthralgias, which gradually became less noticeable. Since that time, she has been asymptomatic, except for a significant flare-up of arthralgia approximately 18 months post-immunization.

Isolation of human lymphocytes. Mononuclear

cells (lymphocytes and monocytes) were prepared from heparinized venous blood by Ficoll-Hypaque gradient centrifugation. The cells at the interface were washed twice in standard medium and were then incubated in RPMI 1640 medium plus 10% fetal calf serum at 35°C in the presence of 0.1% phytohemagglutinin (GIBCO Diagnostics).

Cocultivation of lymphocytes and RK13 cells. RK13 cells were seeded in 60-mm petri dishes (with or without cover slips) and were incubated for 2 days in medium 199 plus 10% fetal calf serum and antibiotic (gentamicin, 20 μg/ml) at 37°C in 5% CO_2. When the cells were subconfluent, lymphocytes (5×10^6) were added to each petri dish, and incubation was continued for several days. No phytohemagglutinin was added to the medium at this stage. At harvest, the medium was retained for infectious virus assay, and the cover slips were washed in phosphate-buffered saline, fixed in acetone, and stored at −20°C. Cultures to be labeled for polyacrylamide gel analysis of intracellular proteins were incubated in Dulbecco minimum essential medium containing 1/5 methionine and 5 μCi of [^{35}S]-methionine per ml for 6 h before being harvested.

Indirect immunofluorescence. Rubella antiserum was prepared in rabbits against high-titer Thomas strain RV, a wild isolate (6) partially purified from the supernatant of infected cells by differential centrifugation. The fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) was obtained from Cappel Laboratories. Incubation with each serum was for 30 min at 20°C, and the cover slips were rinsed with phosphate-buffered saline before they were examined under a Leitz-Wetzlar Diavert inverted microscope.

Assay for infectious virus. A modification of a plaque assay for RV in RK13 cells described by Kouri et al. (9) was used. Microfoci induced by the virus were stained with acridine orange before being counted.

Polyacrylamide gel electrophoresis. Cell pellets were suspended in 0.05 M tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.0)–2% sodium dodecyl sulfate–5% 2-mercaptoethanol–0.005% bromophenol blue–10% glycerol. The solution was heated at 100°C for 1 to 2 min. ^{35}S-labeled polypeptides were then separated in a 9.5% polyacrylamide gel by using the discontinuous buffer system of Laemmli (10). The gels were dried on filter paper and then placed in contact with Kodak RP/XR-1 X-ray film for approximately 10 days. The films were developed in a Kodak RPX-OMAT processor.

Electron microscopy. Cell monolayers were washed with phosphate-buffered saline and fixed with 5% glutaraldehyde in Millonig buffer (pH 7.3) for 1 h. They were postfixed in 1% osmium tetroxide, scraped into distilled water, and pelleted. After dehydration in acetone, they were infiltrated with Spurr epoxy resin and incubated at 70°C overnight to allow hardening. The blocks were sectioned with an ultramicrotome (LKB Instruments, Inc.), and the sections were stained with saturated uranyl acetate and then lead citrate. They were examined on a Phillips EM 300 electron microscope.

Rubella serology and lymphocyte stimulation studies. Rubella hemagglutination inhibition antibody testing and sucrose density gradient fraction studies on serum samples were carried out, as described by Palmer et al. (13), by the Virology Division of the British Columbia Provincial Health Laboratory. Fractions 1 through 3 of the sucrose density gradient contained immunoglobulin of the IgM class, and fractions 4 through 6 contained immunoglobulin of the IgG and IgA classes, as distinguished by standard radial immunodiffusion techniques.

Lymphocyte stimulation assays were performed by using standard [^{3}H]thymidine incorporation adapted to microtiter plates, as previously described (16). Peripheral blood lymphocytes were prepared by Ficoll-Hypaque sedimentation and were suspended in RPMI 1640 supplemented with 10% pretested human blood group AB serum. Serial dilutions of rubella or mumps hemagglutination antigens or their corresponding control antigens (Microbiological Associates) were employed, and stimulation indices were expressed as the ratios of the mean triplicate counts-per-minute determinations for each dilution of viral antigen to the corresponding dilutions of control antigen.

RESULTS

PBL from the patient with rubella-associated arthritis (patient 1) and from a symptom-free control were isolated from peripheral blood by Ficoll-Hypaque gradient centrifugation and were stimulated for 5 days with phytohemagglutinin at 35°C. They were then cocultivated for 3 or 7 days with monolayer cultures of RK13 cells, either in petri dishes or on cover slips, for immunofluorescence studies. By day 3 after cocultivation, cytopathology of the monolayer was apparent in the culture with PBL from patient 1, and on day 7, large numbers of RK13 cells had rounded up and detached from the surface. No such effect was seen with the control samples. On each of these days, cultures were labeled with [^{35}S]methionine for 6 h for protein analysis (the media being retained for infectious virus assay) while further samples were harvested for analysis by electron microscopy and immunofluorescence. The results were compared with those obtained in a previous experiment which used PBL from a normal adult infected (with Thomas strain) or mock-infected in vitro before cocultivation and were analyzed in a similar fashion.

Release of infectious virus. The titers of virus in the supernatant medium were assayed by the formation of microfoci in RK13 monolayers (9) (Table 1). In the culture with lymphocytes infected in vitro, $>10^7$ microfocus-producing units per ml were detected 3 days after cocultivation whereas the medium from the patient 1 lymphocyte-RK13 coculture had a titer of 10^4 microfocus-producing units per ml. This titer increased to 4.5×10^6 microfocus-producing units per ml on day 7, by which time the culture

TABLE 1. *Titer of infectious virus in the supernatant medium of lymphocytes cocultivated with RK13 cells*[a]

Determination	Infectious virus titer (MPU[b]/ml)	
	Day 3	Day 7
Expt 1		
Mock-infected lymphocytes, RK13	0	NT[c]
Infected lymphocytes, RK13	3.2×10^7	NT
Expt 2		
Lymphocytes from patient 1, RK13	1.2×10^6	4.5×10^6
Control lymphocytes, RK13	0	0

[a] Assayed by the production of microfoci.
[b] MPU, Microfocus-producing unit.
[c] NT, Not tested.

TABLE 2. *RV antigens in the cytoplasm of RK13 cells cocultivated with lymphocytes*[a]

Determination	Cytoplasmic fluorescence	
	Day 3	Day 7
Expt 1		
Mock-infected lymphocytes, RK13	−	NT[b]
Infected lymphocytes, RK13	+++	NT
Expt 2		
Lymphocytes from patient 1, RK13	+/−	+
Control lymphocytes, RK13	−	−

[a] Detected by indirect immunofluorescence, using antisera against purified RV and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG.
[b] NT, Not tested.

with lymphocytes infected in vitro had been completely lysed. The mock-infected culture and the lymphocytes from a symptom-free adult showed no detectable virus when assayed by this technique. The production of microfoci by medium from the patient 1 culture was subsequently shown to be greatly reduced by incubation of the medium (day 7) with anti-RV antiserum for 10 min before assay of microfocus production (4.5×10^6 microfocus-producing units per ml reduced to 2.7×10^3 microfocus-producing units per ml), which proved that the infectious agent was RV. Cocultivation of the lymphocytes with a permissive cell line appears to be an essential step in virus reactivation, since no infectious virus or viral proteins were detected at any stage in PBL cultured alone (not shown).

Immunofluorescence studies. The expression of RV antigens in the cocultures was examined by indirect immunofluorescence, using antisera against structural components of RV. The prebleed serum and RV antiserum were adsorbed thoroughly against RK13 cells before use and were used in conjuction with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Table 2). The cocultures of PBL infected in vitro showed widespread cytoplasmic fluorescence involving >80% of the cells in the monolayer. In comparison, cytoplasmic fluorescence in the patient 1 lymphocyte-RK13 cultures was much less intense, even by day 7 post-cocultivation, and only 10% (day 3) to 35% (day 7) of the cells scattered throughout the monolayer were involved. In no case did the prebleed serum give any fluorescence, and negligible amounts of background fluorescence were found in the control (mock-infected) cultures with anti-RV antiserum.

Viral proteins. The expression of individual viral proteins in the cocultivated cells was examined by polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled cell extracts. Detection of viral proteins is difficult because host translation is not inhibited in RV-infected RK13 cells. However, RV polypeptides in both RK13 cells (2) and in human lymphocyte subpopulations (2a) have previously been well characterized, and these studies have greatly aided us in determining the degree of virus expression occurring against a high background of cellular protein synthesis. The virus-induced polypeptides in the cocultured cells are indicated with arrows and designated by their molecular weights (10^3) in Fig. 1. Six viral polypeptides are distinguishable in the RK13 cells cocultivated with Thomas strain-infected lymphocytes (Fig. 1, lane a) and also in the patient 1 lymphocyte-RK13 culture, although, with the exception of p75, the polypeptides were found in smaller amounts in the lymphocyte-RK13 culture. In particular, p66, which is probably a precursor of one or more structural proteins (2), was greatly reduced in the patient 1 lymphocyte-RK13 culture, a result in accordance with the lower titers of the infectious virus produced in these cells.

Electron microscopy. Thin sections of cells fixed with glutaraldehyde-osmium and stained with uranyl acetate and lead citrate were examined with a Phillips EM 300 electron microscope microscope (Fig. 2). Large vacuolated areas were apparent in the cytoplasms of both the infected and patient 1 PBL cocultures, and virus particles similar in size and appearance to those observed by Edwards et al. (4) in RV-

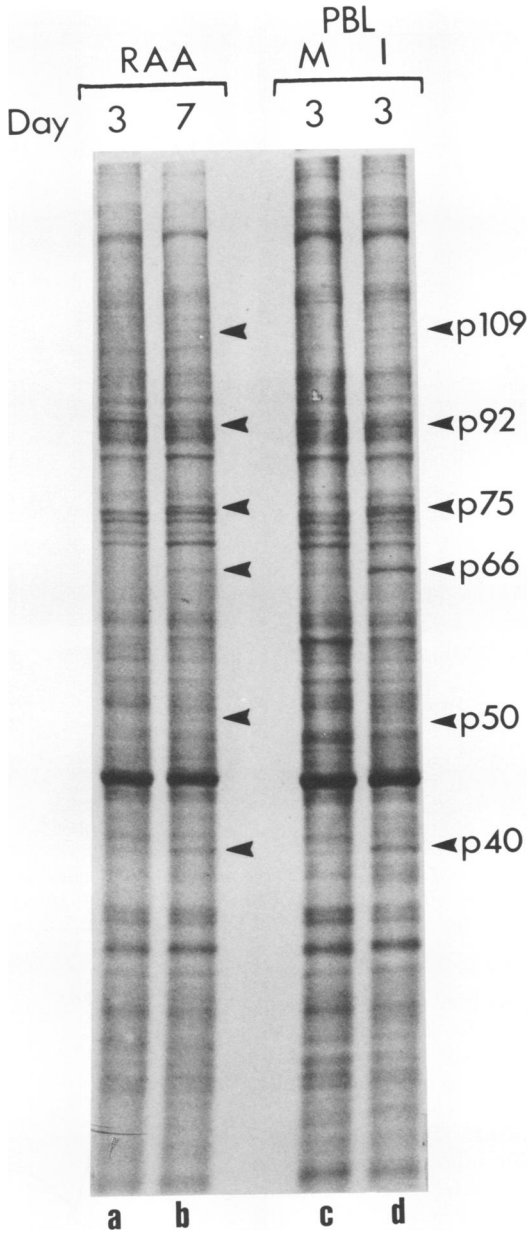

FIG. 1. *Autoradiogram of a 9.5% slab gel showing the spectrum of labeled polypeptides in cocultures of RK13 cells with lymphocytes (d) infected (I) or (c) mock-infected (M) with RV in vitro or obtained from the patient with rubella-associated arthritis (RAA) (a and b). Viral polypeptides are indicated with arrows and are designated according to their molecular weights (10^3).*

infected BHK-21 cells could be seen in each case. In Fig. 2A (patient 1 PBL-RK13 culture), two vacuoles partially surrounded by membranous structures of the endoplasmic reticulum and containing several virus particles (50 to 75 nm) and some aberrant virus structures can be seen. The virus particles were generally found in close association with membranous elements within the cytoplasm, and no budding from the plasma membrane was observed. This was also true of the samples infected in vitro (Fig. 2B). However, these cells appeared to contain less proliferating membrane structures than the patient 1 PBL-RK13 coculture. Most virions in our

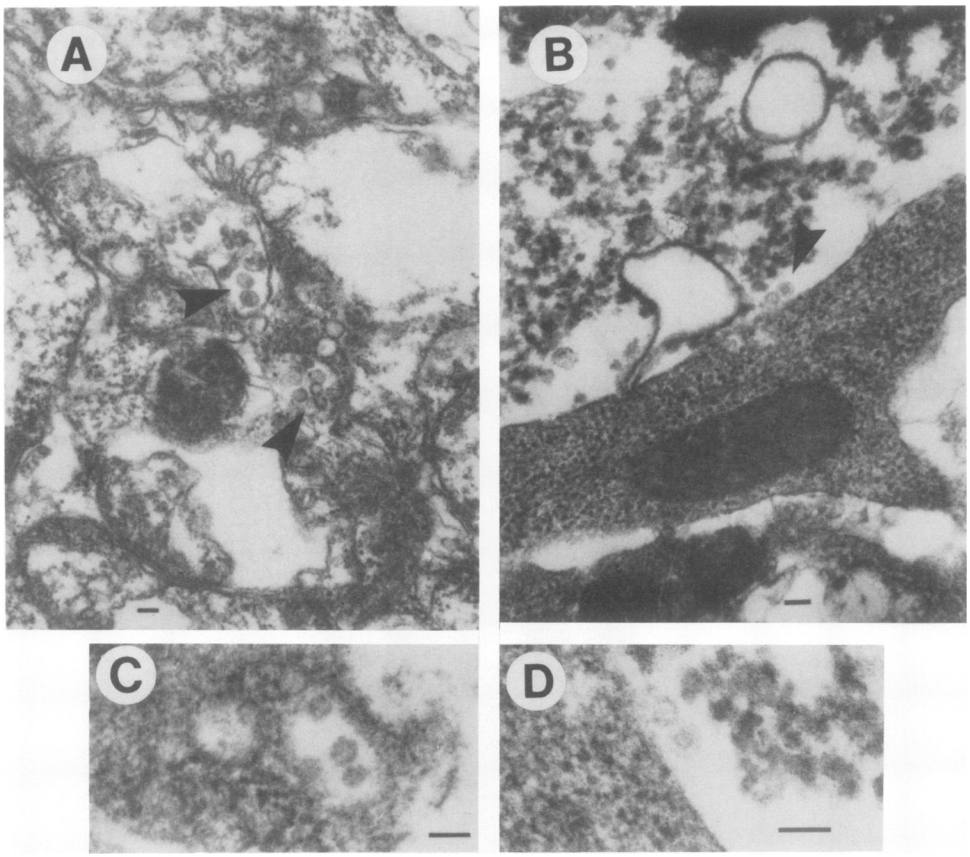

FIG. 2. *Electron micrographs of (A) patient 1 lymphocyte-RK13 coculture showing vacuolated areas containing particles 50 to 75 nm. (B) RV-infected PBL-RK13 coculture showing a large area of cytopathic effect and two distinct virus particles (arrows). (C and D) Higher magnification of particles found in (A) and (B), respectively.*

preparations had lightly stained cores, although an occasional dark core (Fig. 2B, arrow) was seen. At high magnification, the unit membrane structures of the envelope could be distinguished (Fig. 2C and D).

Immunological studies. (i) Nonspecific. Assessment of nonspecific immunological reactivity, including lymphoproliferative responses to phytohemagglutinin, pokeweed mitogen, and mumps antigen (Fig. 3 and 4), IgM, IgG, and IgA levels, and C3 complement levels was completely within normal limits. In addition, antinuclear antibody and rheumatoid factor determinations have been consistently negative on repeated testing.

(ii) Rubella specific. Rubella serological studies both before and at sequential time intervals after HPV-77 DE/5 immunization are outlined in Table 3. These studies document seroconversion by the RV hemagglutination inhibition technique after immunization and indicate a sustained RV hemagglutination inhibition titer of 1:64 at 27 months post-immunization. The RV hemagglutination inhibition antibody of the IgM class was detected 4 and 6 weeks but not 8 weeks post-immunization by using sucrose density gradient fractionation techniques.

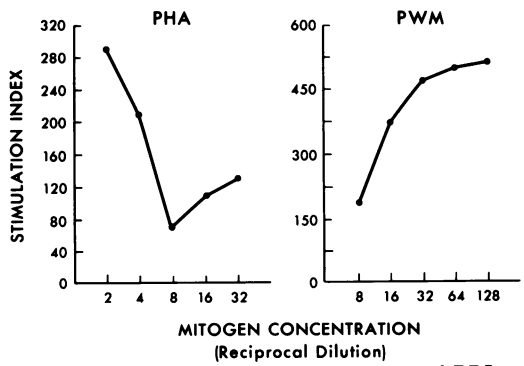

FIG. 3. *Lymphoproliferative responses of PBL to phytohemagglutinin (PHA) or pokeweed mitogen (PWM) 27 months after HPV-77 DE/5 immunization.*

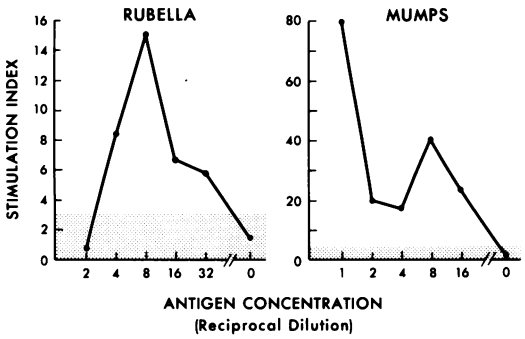

FIG. 4. *Lymphoproliferative responses of PBL to rubella hemagglutination antigen or mumps hemagglutination antigen 27 months after HPV-77 DE/5 immunization. Stippled areas represent negative (stimulation index, <3.0).*

TABLE 3. *Rubella hemagglutination inhibition titers*

Time post-immunization	Titer		
	Whole serum	Sucrose density gradient fraction	
		IgM	IgG
0	<1:8	<1:8	<1:8
1 wk	<1:8	<1:8	<1:8
2 wk	<1:8	<1:8	<1:8
4 wk	1:32	1:8	<1:8
6 wk	1:32	1:8	1:16
8 wk		<1:8	
27 mo	1:64		

Rubella lymphocyte stimulation studies carried out in parallel with virus rescue studies are presented in Fig. 4 and demonstrate high levels of specific sensitization to RV in PBL.

DISCUSSION

The results show the presence of RV in the PBL of a 26-year-old female with recurrent episodes of rubella-associated arthritis and arthralgia 27 months after immunization with HPV-77 DE/5 vaccine. The detection of the virus required incubation of PBL with a permissive cell line in culture, which indicated that virus reactivation or "rescue" was required. This suggests that a very small proportion of cells were harboring the virus in vivo or that some restriction on viral replication occurs in PBL in vivo, perhaps similar to that found in isolated B lymphocytes (2a). Since we were only able to detect the virus after reactivation in vitro had occurred, it is not surprising that complete virus expression and the production of infectious virus were observed. This does not tell us the state of the viral genome or the degree of virus expression occurring during in vivo persistence.

It is interesting that the detection of RV persistence in this individual was not associated with a lack of immune responsiveness to the virus with a state of generalized immune suppression. The serological response to RV was unremarkable in both the transient production of IgM antibody and the sustained RV hemagglutination inhibition titer of 1:64 over 2 years postvaccination. In addition, lymphoproliferative responses to mitogens were normal and were elevated to RV antigens, as previously reported in other individuals with recurrent rubella-associated arthritis (5). These findings contrast with the observation that acute infection with wild or vaccine strains of RV is followed by a transient depression of mitogen-induced lymphoproliferation, which is associated temporally with the recovery of infectious RV from PBL (1; J. K. Chantler and A. J. Tingle, submitted for publication). However, in cases of congenital rubella, virus isolation associated with a lack of immune suppression and with normal or slightly elevated levels of sensitization to RV has also been demonstrated (14). Thus, it appears that long-term persistence of RV in lymphocyte populations is not necessarily associated with any drastic alteration in immune function, although subtle alterations in immune regulation may occur. On the other hand, periodic reactivation of RV from persistently infected PBL, with the resulting formation of virus-antibody immune complexes which may accumulate in the joints and initiate a local inflammatory response, provides one explanation for the pathogenesis of rubella-associated arthritis.

During the last year, we have examined a number of other cases of rubella-associated arthritis. In no case has such clear-cut evidence of RV isolation been obtained. However, a considerable degree of cytopathology and cell lysis has always been observed in the cocultivation of PBL from patients with RK13 cells. Cytopathology and cell lysis have not been seen in the cocultivation of PBL of normal individuals or of several children (9 to 15 years old) with congenital rubella syndrome. In addition, electron microscopic examination of the cultures has shown large vacuolated areas typical of virus-infected cells containing what appears to be aberrant virus particles. At present, we are attempting to rescue infectious RV from such cases with a variety of inducing agents and with alterations of the incubation temperature, in case a temperature-sensitive mutant is involved. The results of this study will be published in due course.

ACKNOWLEDGMENTS

We thank Theresa Sapp and Stephen Hosford for excellent technical assistance.

This project was supported by the Arthritis Society, Canada, and by the Medical Research Council of Canada.

LITERATURE CITED

1. **Buimovici-Klein, E, and L. Z. Cooper.** 1979. Immunosuppression and isolation of rubella virus from human lymphocytes after vaccination with two rubella vaccines. Infect. Immun. **25**:352–356.
2. **Chantler, J. K.** 1979. Rubella virus: intracellular polypeptide synthesis. Virology **98**:275–278.

2a. **Chantler, J. K., and A. J. Tingle.** 1980. Replication and persistence of RV in human lymphocyte populations. J. Gen. Virol. **50**:317–328.

3. **Cooper, L. Z., P. R. Siring, H. J. Weiss, B. A. Matters, and S. Krugman.** 1969. Transient arthritis after rubella vaccination. Am. J. Dis. Child. **118**:218–225.
4. **Edwards, M. R., S. M. Cohen, M. Bruno, and R. Deibel.** 1969. Micromorphological aspects of the development of rubella virus in BHK-21 cells. J. Virol. **3**: 439–444.
5. **Ford, D. K., and A. J. Tingle.** 1980. Cell-mediated immune response in patients with recurrent arthritis following rubella immunization. J. Rheumatol. **7**:225–230.
6. **Hart, H., and B. P. Marmion.** 1977. Rubella virus and rheumatoid arthritis. Ann. Rheum. Dis. **36**:3–12.
7. **Hildebrandt, H. M., and H. F. Massab.** 1966. Rubella synovitis in a one year old patient. N. Engl. J. Med. **274**:1428–1430.
8. **Kantor, T. G., and M. Tanner.** 1962. Rubella arthritis and rheumatoid arthritis. Arthritis Rheum. **5**:378–383.
9. **Kouri, G., A. Aguitera, P. Rodriguez, and M. Korolev.** 1974. A study of microfoci and inclusion bodies produced by rubella virus in the RK13 cell line. J. Gen. Virol. **22**:73–80.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Martenis, T. W., J. H. Bland, and C. A. Phillips.** 1968. Rheumatoid arthritis after rubella. Arthritis Rheum. **11**:683–685.
12. **Ogra, P. L., and J. K. Herd.** 1971. Arthritis associated with induced rubella infection. J. Immunol. **107**:810–813.
13. **Palmer, D. F., K. L. Herrmann, R. E. Lincoln, M. V. Hearn, and J. M. Fuller.** 1970. A procedural guide to the performance of the standardized rubella hemagglutination test, p. 1–43. *In* Immunology series no. 2. Centers for Disease Control, Atlanta.
14. **Simons, M. J., and M. G. Fitzgerald.** 1968. Rubella virus and human lymphocytes in culture. Lancet **ii**:937.
15. **Simons, M. J., and I. Jack.** 1968. Lymphocyte viremia in congenital rubella. Lancet **ii**:953–954.
16. **Tingle, A. J., D. K. Ford, G. E. Price, and G. D. Kettyls.** 1979. Prolonged arthritis in identical twins following rubella immunization. Ann. Intern. Med. **90**: 203–204.
17. **Weibel, R. E., J. Stokes, E. B. Bunyak, and M. R. Hilleman.** 1969. Live rubella vaccines in adults and children. Am. J. Dis. Child. **118**:226.
18. **Wolinsky, J. S., P. C. Dau, E. Buimovici-Klein, J. Nednick, B. O. Berg, P. B. Lang, and L. Z. Cooper.** 1979. Progressive rubella panencephalitis: immuno-virological studies and results of isoprinosine therapy. Clin. Exp. Immunol. **35**:397–404.
19. **Yanez, J. E., G. R. Thompson, W. M. Mikkelson, and L. E. Bartholomew.** 1966. Rubella arthritis. Ann. Intern. Med. **64**:772–777.