

REPLICATION OF MEASLES VIRUS IN HUMAN LYMPHOCYTES

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Measles is a common disease of childhood that often causes complications in the central nervous system (CNS)¹. Measles virus antigens are expressed in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) during acute measles infection, and also in PBMC of patients with subacute sclerosing panencephalitis (1), which is a chronic measles virus infection of the CNS. Because lymphocytes can survive long times in the body, it has been suggested (2) that measles virus might be modulated and carried to CNS inside these cells. Measles infection causes impairment of delayed cutaneous reactivity (3) and suppression of lymphocyte responses to mitogens (4). Depressed immunological reactivity can expose patients to diseases caused by other infectious agents. On the other hand, acute measles infection is characterized by polyclonal activation of B cells (5).

Measles virus is able to infect peripheral blood leukocytes *in vitro*. Productive infection has been shown to occur in T and B cells and, to a lesser extent, in monocytes, whereas mature polymorphonuclear cells fail to support the replication of measles virus (2, 6). Moreover, subpopulations of T cells with both suppressor/cytotoxic and helper phenotypes can be infected *in vitro* (7). The virus may preferentially infect cells responsible for helper functions, because defective production of helper factors has been described (8). The ratio between OKT8⁺/OKT4⁺ cells remains, however, normal during acute infection (5). The infection is relatively silent in unstimulated leukocytes. However, the natural killer cell activity and the Ig-synthesizing capacity of these cells is impaired (9), and the cells are known to produce virus-induced interferon (10). The stimulation of the PBMC by individual structural polypeptides of measles virus causes lymphocyte proliferation in high-responder individuals (11). When the PBMC are stimulated with PHA after *in vitro* infection, they lose their stimulatory response, as measured by [³H]thymidine incorporation (our unpublished results). The PHA-stimulated cells, however, start to synthesize high levels of extracellular virus, and to express increased amounts of viral antigens on their surface (12). A clear increase in virus production can also be achieved by antiinterferon treatment of the infected cells (10).

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; CNS, central nervous system; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCID₅₀, tissue culture infective dose.

In our study, we have characterized the replication of measles virus in PBMC both in vivo and in vitro.

Materials and Methods

Isolation and Cultivation of PBMC. PBMC were isolated from the blood of 26 measles patients (1–30 d after the appearance of the rash), and from eight healthy young adults by Ficoll-Isopaque (Ficoll-Paque, Pharmacia Fine Chemicals, Uppsala, Sweden) gradients (13). The cells and the serum specimens were stored at -70°C until tested. The diagnosis of measles was based on the presence of antimeasles IgM in the serum of the patients (14).

The isolation and enrichment of cell populations were carried out as described by Eskola et al. (15). T lymphocytes were rosetted with 2-aminoethylisothiuronium bromide-treated sheep red blood cells, and separated from other mononuclear cells by Ficoll-Isopaque centrifugation. A monocyte-enriched cell population was obtained by collecting plastic-adherent cells. The population of nonadherent cells was considered as enriched B cells. OKT4⁺- and OKT8⁺-depleted cells were prepared from T cells by specific, complement-mediated lysis with monoclonal antibodies against these T cell subpopulations (15). For in vitro studies, the mononuclear cells were infected with a wild type strain of measles virus (16) with a multiplicity of 0.1–1. Infected and control cells (10^6 cells/ml) were cultured either unstimulated, or stimulated with PHA (10 $\mu\text{g}/\text{ml}$; Difco Laboratories, Detroit, MI) and incubated for various times at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gibco Europe, Glasgow, United Kingdom) and 50 $\mu\text{g}/\text{ml}$ gentamicin (17). The cells and the supernatants were harvested on days 0–5, 7, and 10 postinfection, and stored -70°C until tested.

Infectivity Titration. The amount of extracellular virus was measured by incubation of serial 10-fold dilutions of supernatants on confluent monolayers of Vero cells. Each dilution was assayed in triplicate. The results were obtained after a 10 d-incubation period, and the amount of measles virus was expressed as tissue culture infective doses (TCID₅₀)/ml.

Electron Microscopy. Uninfected and in vitro-infected PBMC were fixed with 5% glutaraldehyde in S-collidine HCl buffer, pH 7.4, and postfixed in buffered 2% osmium tetroxide. The fixed cells were dehydrated in washes of ethanol, followed by propylene oxide. The cells were then infiltrated in a 1:1 mixture of Ladd's resin and propylene oxide, and embedded in the resin. Sections were cut with an ultramicrotome and stained with 12.5% uranyl acetate and 0.25% lead citrate, and examined in a Philips EM 410 electron microscope.

Immunofluorescence. For surface immunofluorescence, the PBMC were fixed with 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, at 25°C for 15 min. For intracellular staining, fixed cells were further permeabilized with 0.05% Triton X-100 in phosphate-buffered saline (PBS), pH 7.2, at 25°C for 30 min (18). The fixed cells were incubated for 30 min at 37°C with rabbit antimeasles serum diluted 1:500; the cells were then washed three times with PBS and incubated for another 30 min at 37°C with sheep anti-rabbit Ig conjugated with fluorescein isothiocyanate (Wellcome, Beckenham, United Kingdom). The fluorescence was examined with a Leitz Dialux 22 microscope.

Dot-blotting. The amount of measles virus-specific proteins in PBMC was determined by dot-blotting (19). A total of 5×10^4 cells/specimen were washed twice with PBS, and then solubilized with 2% sodium dodecyl sulfate (SDS) in 0.1 M Tris HCl buffer, pH 6.8, and spotted onto a nitrocellulose filter (0.45 μm , BA85, Schleicher and Schuell Co., Dassel, Federal Republic of Germany). The remaining protein-binding capacity was blocked by incubating the filter for 2 h at room temperature in PBS containing 3% bovine serum albumin (BSA) and 1% FCS. The filters were then incubated overnight at 4°C with rabbit antimeasles serum diluted 1:500. The antigen-bound antibodies were detected by incubating the filters with ^{125}I -labelled anti-rabbit IgG (10^4 cpm/ml) in PBS containing 3% BSA, 1% FCS, 0.5% Triton X-100, 0.5% deoxycholate. The bound radioactivity was revealed both by autoradiography and gamma counting. Known amounts (25 μg , 1 μg , 100 ng, 10 ng) of purified measles virus proteins (16) were used as standards.

SDS-polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting. The presence of measles virus structural proteins in the cells was analyzed by 10% SDS-PAGE (20), and by immunoblotting (21). A total of 10^5 cells were washed twice with PBS, lysed with 2% SDS, and analyzed by electrophoresis. After the electrophoresis, the polypeptides were transferred to nitrocellulose (0.22 μm , BA-85, Schleicher and Schuell Co.) and the virus-specific antigens were detected with rabbit antimeasles serum followed by horseradish peroxidase-conjugated anti-rabbit IgG, and the substrate (200 $\mu\text{g}/\text{ml}$ of 3-amino-9-ethylcarbazole, 1 μl H_2O_2 , 50 mM sodium acetate buffer, pH 5).

Spot Hybridization. A modification of an earlier-described (22) method was used. The cells were treated for 1 h at 37°C with proteinase K (E. Merck AG, Darmstadt, Federal Republic of Germany). The solution was then adjusted to contain 0.9 M NaCl and 0.09 M sodium citrate, and spotted onto nitrocellulose (0.45 μm , BA85, Schleicher and Shuell Co.). The filters were baked for 2 h at 80°C, and prehybridized for 2 h at 42°C in 50% formamide, 50 mM Hepes, pH 7.4, 0.45 M NaCl, 0.045 M sodium citrate, Denhardt's solution (23), 0.1% SDS, 250 $\mu\text{g}/\text{ml}$ of yeast RNA, and 100 $\mu\text{g}/\text{ml}$ of denatured herring sperm DNA. The probe was prepared by a random primed reaction (24), using measles virus genomic RNA as a template, and α -[^{32}P]deoxycytidine 5'-triphosphate as the isotope. The probe, sp act $\sim 10^7$ cpm/ μg was denatured, and directly added to the prehybridization solution, then incubated overnight at 42°C. The filter was washed in 0.3 M NaCl and 0.03 M sodium citrate, containing 0.5% SDS, at 65°C for 1 h, and the radioactivity was detected either by autoradiography overnight, or by scintillation counting.

Results

No detectable amount of extracellular infectious measles virus was released from in vitro-infected, unstimulated PBMC during the 10-d culture period (Fig. 1A). On the other hand, PHA stimulation induced production of extracellular virus, and the maximal titer (10^4 TCID₅₀) was reached 3 d postinfection.

Immunofluorescence and Electron Microscopy. The intensity of measles virus-specific fluorescence (Fig. 2A) in individual in vitro-infected PBMC increased during the culture period, indicating active measles virus protein synthesis. The proportional amount of positive cells, however, remained at the range of 20–25% (Fig. 1B). Electron microscopic examination showed typical paramyxovirus nucleocapsid structures in the cytoplasm of the unstimulated PBMC (Fig. 3B), which confirms that measles virus replicates in unstimulated cells, and virus-specific fluorescence did not originate from inoculated material. PHA stimulation induced a high increase in the percentage of measles virus antigen-positive cells (Fig. 1B and Fig. 2B).

Measles Virus Polypeptides in PBMC. No clear increase was observed in the total amount of measles virus-specific protein synthesis in unstimulated, in vitro-infected PBMC by dot-blotting (Fig. 1C) as was expected because of the low proportion of positive cells in immunofluorescence (Fig. 1B). Accordingly, not more than two major virus-specific polypeptides, hemagglutinin (79 kilodaltons [kD]), and nucleoprotein (60 kD), were detected by immunoblotting in unstimulated cells (Fig. 4A). PHA-stimulated cells supported the synthesis of large amounts of measles virus-specific proteins (Fig. 1B) with polypeptide patterns similar to those seen in lytically infected cells (Fig. 4D).

Measles Virus RNA in PBMC. The amount of measles virus RNA in the cells was determined by spot hybridization using a probe complementary to viral genomic RNA (Fig. 6, A and B). In the unstimulated PBMC, the amount of viral RNA increased slowly during the culture period. However, a twofold difference

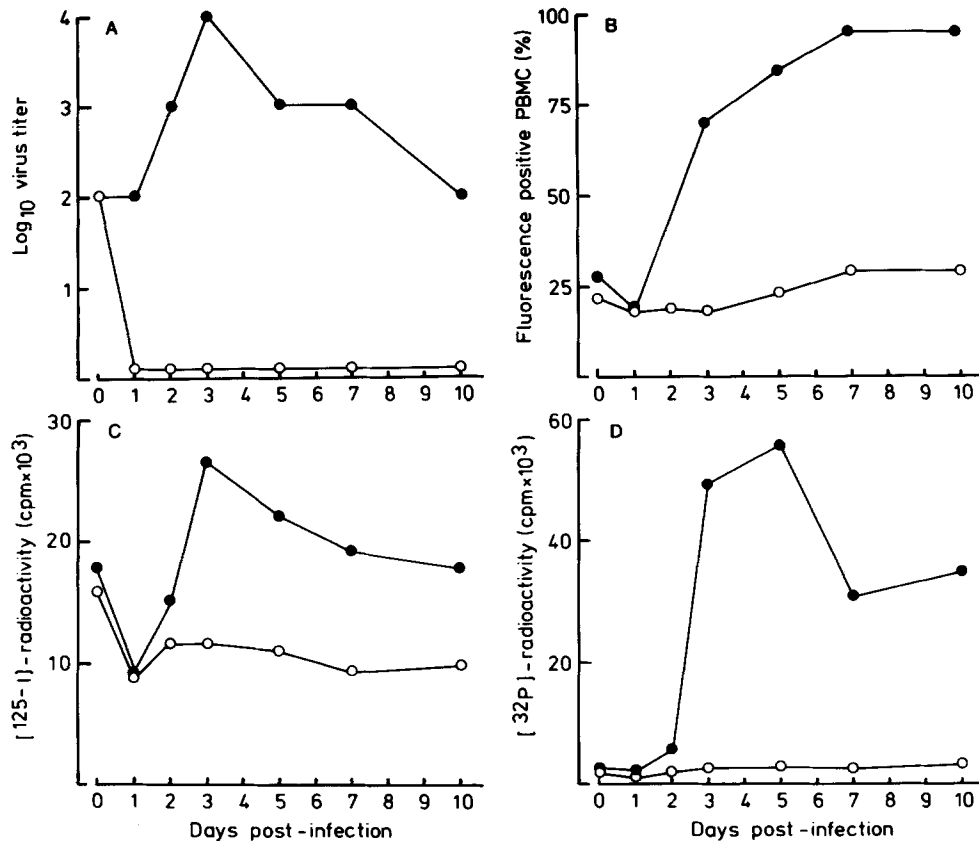


FIGURE 1. Replication of measles virus in PBMC. The cells were infected with measles virus, and cultured either unstimulated (○), or stimulated with PHA (●). The amount of extracellular virus (A), the proportional amount of antigen-positive cells (B), the quantity of measles virus-specific proteins (C) and RNA (D) were determined at 0, 1, 3, 5, 7, and 10 d postinfection.

was noticed between days 3 and 7 postinfection (Fig. 5A). PHA stimulation caused similar increase in RNA synthesis (Fig. 1D) as was seen in production of extracellular virus, and in virus-specific protein synthesis (Fig. 1, A and B).

Replication of measles virus RNA was repeatedly observed in enriched populations of unstimulated T and B cells, and to a lesser extent in monocytes (Fig. 5, D, C, and B, respectively). The removal of subpopulations with helper/inducer (OKT4⁺) or suppressor/cytotoxic (OKT8⁺) phenotypes from total T cells did not decrease the replication (Fig. 5, E and F).

Virus Components in PBMC of Measles Patients. PBMC were isolated from 26 patients with acute measles infection. When tested by spot hybridization, 16 patients had detectable amounts of measles virus RNA in their cells. Results of serial specimens from three positive patients are presented in Fig. 6D. The controls, consisting of eight healthy adults with natural histories of measles in childhood, were all negative, by hybridization.

The test was most often positive during the first week of illness, but the results in serial specimens were widely variable between individuals.

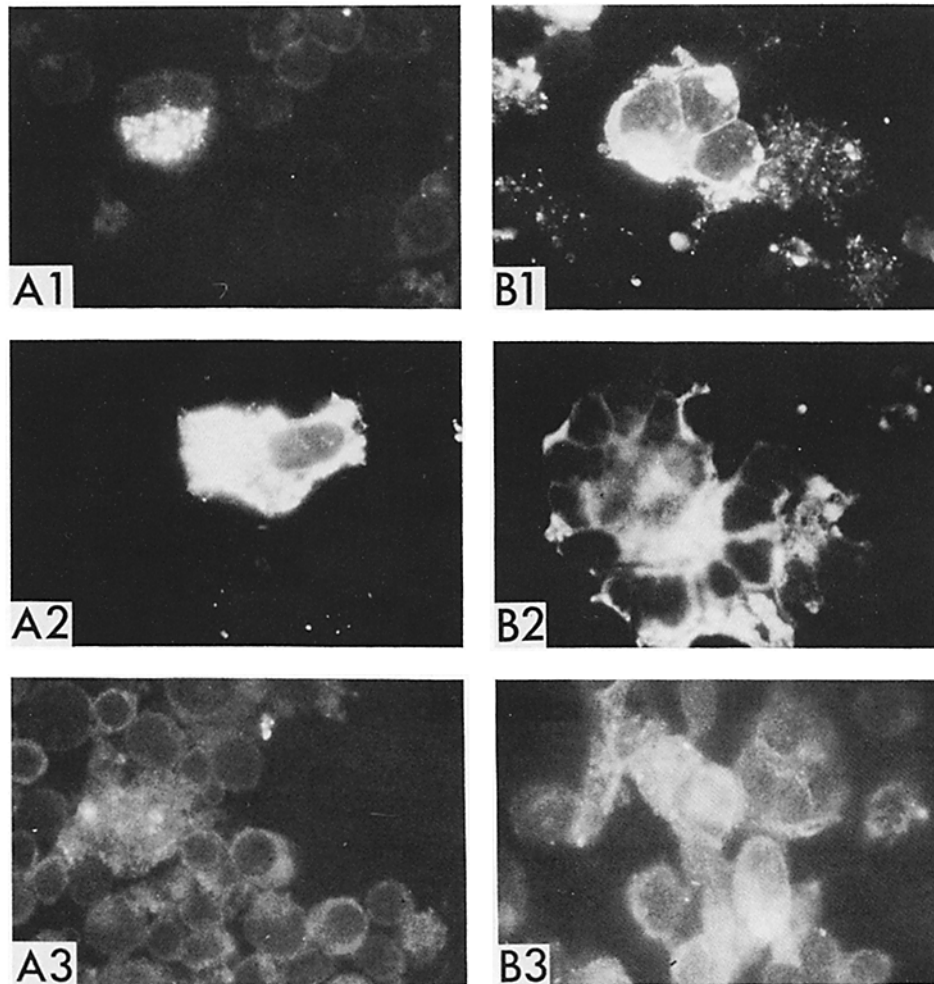
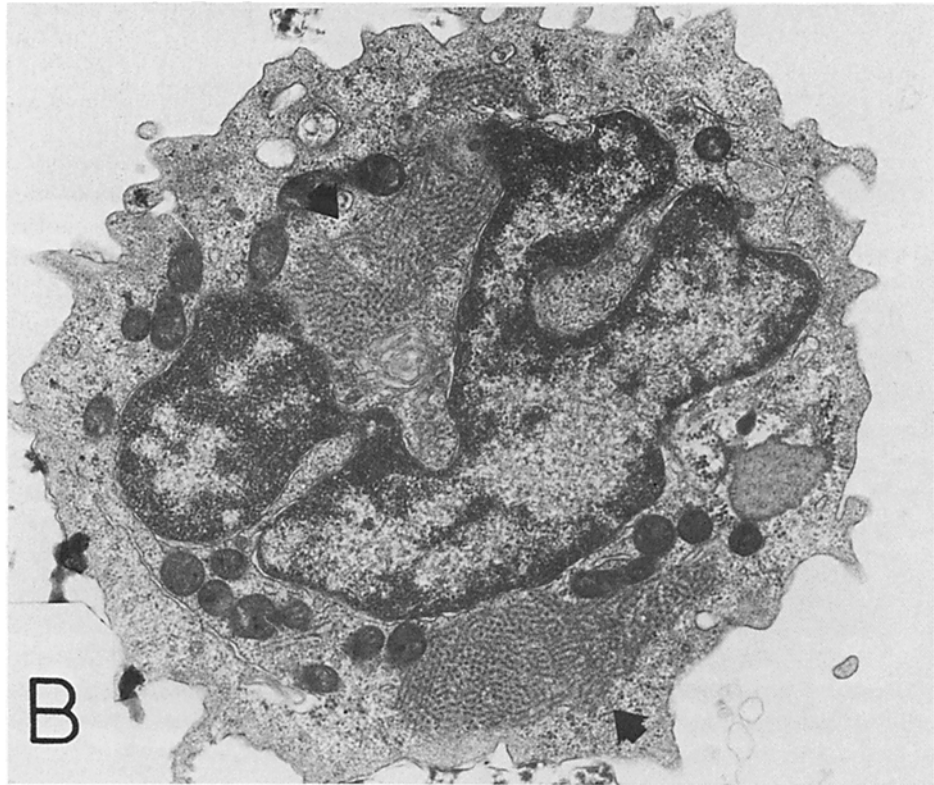
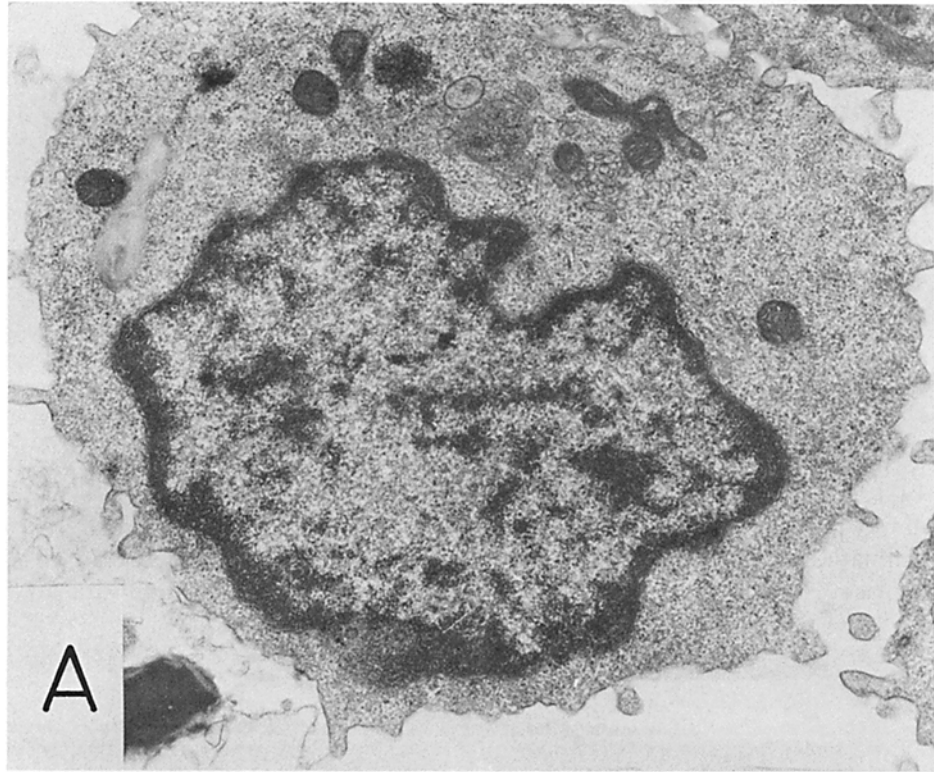


FIGURE 2. Indirect immunofluorescence staining of *in vitro*-infected PBMC. The unstimulated (A) and PHA-stimulated (B) PBMC were stained either with rabbit antimeasles serum (1, 2), or preimmune serum (3). Prior to staining, the cells had been fixed in 3% paraformaldehyde (1), and, in addition, permeabilized with 0.05% Triton X-100 (2, 3).

Immunoblotting analysis of PBMC from three measles patients with strongly positive results in spot hybridization showed the presence of only one major virus-specific polypeptide, nucleoprotein (60 kD) (Fig. 4C). The proportional amount of the other major protein, hemagglutinin (79 kD) was clearly lower than in the *in vitro*-infected PBMC.

Discussion

Measles virus infection of PBMC *in vivo* and *in vitro* disturbs mitogen response (4), natural killer cell activity, and Ig synthesis in these cells (9). Because the infection in unstimulated PBMC is relatively silent, i.e. the production of virus and its structural components is low, the analysis of viral macromolecular synthe-



sis has been difficult. The availability of new, sensitive, and specific methods has made it possible to characterize the infection in more detail.

The low replication rate of measles virus in unstimulated PBMC can be explained either by infection of only certain subpopulations, as shown recently for human T cell leukaemia (lymphotropic) virus III (25), or by a replication block in macromolecular synthesis. In the latter case, it is possible that the replication of viral RNA is already restricted, or that the viral transcription, translation, or assembly is disturbed. To study the different levels of viral replication, we analyzed the amount of viral RNA, polypeptides, and infectious virus of in vitro-infected, unstimulated, or PHA-stimulated PBMC during a culture period of 10 d.

The amount of immunofluorescence-positive PBMC (20–25%) remained relatively constant through the whole culture period. The low proportion of positive cells, together with the lack of infectious virus in culture supernatants, indicates that no productive infection cycle occurs. However, in electron microscopic examination, typical measles virus nucleocapsid structures were seen in the cytoplasm of the infected cells. This confirms that measles virus-specific RNA and proteins are synthesized also in unstimulated PBMC.

The replication of viral RNA was studied by a hybridization assay. In addition to the genomic minus-strand RNA, the complementary DNA probe also recognizes the viral messenger RNA of positive polarity. This is due to the presence of both plus and minus strands in paramyxovirions. The assay showed that active replication of virus RNA occurs in unstimulated cells in vitro. To study the presence of measles virus RNA in vivo, PBMC were isolated from patients with acute measles infection. Two of the specimens were positive in the hybridization test even after 3 wk from the onset of the rash. This indicates that measles virus genetic information may remain in the cells long enough to be modified and transported to other tissues.

To study whether the measles virus infection of PBMC in vitro would be restricted to certain subpopulations, we used nucleic acid hybridization to measure the amount of viral RNA during the culture period. The results confirm the earlier observations (2, 6) that both T and B cells, but in a lesser extent monocytes, can support the replication of measles virus. The depletion of OKT4⁺ or OKT8⁺ subsets of T cells did not change the course of infection. This method is known to reduce the amount of the depleted cell population to <1% of the surviving cells. Thus, the 20% proportion of PBMC showing positive fluorescence cannot represent any of the defined subsets studied here.

The dot-blotting results indicate that the synthesis of viral polypeptides is low, but in immunoblotting, at least nucleocapsid and hemagglutinin proteins were detected. However, the proportional amount of the immunoreactive hemagglutinin in PBMC of measles patients was clearly lower compared to its amount in in vitro-infected PBMC. Our preliminary results with interferon-treated, in vitro-infected PBMC indicate a similar decrease in the amount of the hemagglu-

FIGURE 3. Morphological appearance of uninfected (A) and measles virus-infected (B), unstimulated PBMC. The arrows show the presence of typical measles virus nucleocapsid structures.

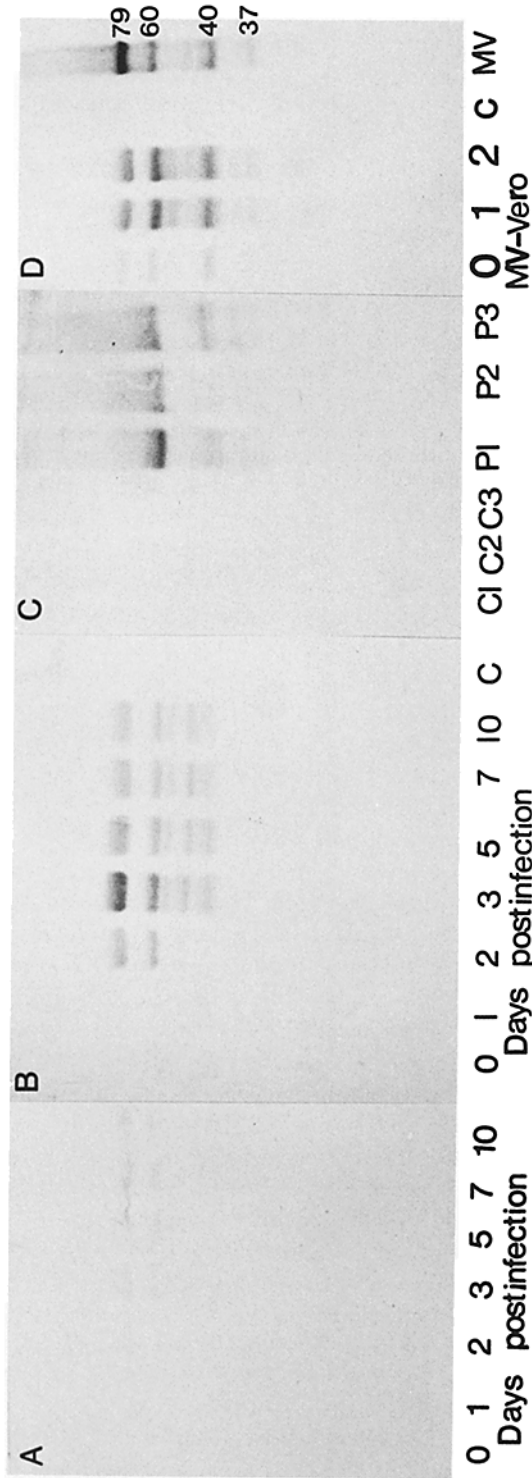


FIGURE 4. Immunological detection of measles virus-specific polypeptides in vitro-infected, unstimulated (A), or PHA-stimulated (B) PBMC, in PBMC of controls (C, lanes C1-C3), and of measles patients (C, lanes P1-P3), and in lytically infected Vero cells (D). Lane C, uninfected control cells, lane MV, purified measles virus. The polypeptides were analyzed by SDS-PAGE, transferred to nitrocellulose, and detected with rabbit anti-measles serum and horseradish peroxidase-conjugated anti-rabbit antibodies.

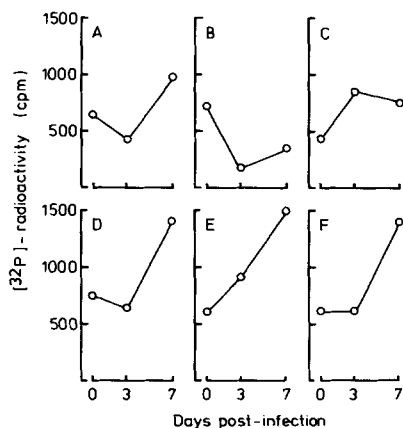


FIGURE 5. Replication of measles virus RNA in *in vitro*-infected, unstimulated subpopulations of PBMC, detected by spot hybridization. (A) unfractionated PBMC; (B) monocytes; (C) B cells; (D) T cells; (E) OKT4⁺-depleted T cells; (F) OKT8⁺-depleted T cells.

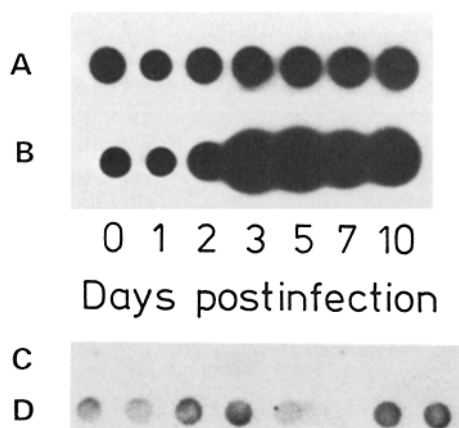


FIGURE 6. Detection of measles virus RNA in *in vitro*- and *in vivo*-infected PBMC by spot hybridization. (A) unstimulated PBMC infected *in vitro*; (B) PHA-stimulated PBMC infected *in vitro*; (C) PBMC from eight healthy adults; (D) Serial PBMC specimens from patients with acute measles (patient I, spots 1 and 2; II, 3-5; III, 6-8).

tinin protein.

Our results favor the possibility that measles virus replication in *in vitro*-infected PBMC may be complete, but limited. PHA stimulation, however, causes a productive infection cycle, indicating that effective replication requires components that are present only in activated cells. This may explain the phenomenon that only a restricted population of PBMC supports the synthesis of viral components. In PBMC isolated from measles patients, defective synthesis of the hemagglutinin protein is observed, which may be due to effects of circulating factors such as interferons.

Summary

Replication of measles virus was restricted in human peripheral blood mononuclear cells (PBMC). However, in *in vitro*-infected, unstimulated cells, active

synthesis of viral RNA and proteins occurred, while the release of infectious virus could not be detected. Stimulation with PHA caused a productive infection cycle comparable to the lytic infection. Replication of viral RNA was demonstrated in both T and B cells, and in both OKT4⁺- and OKT8⁺-depleted T cell subsets. The presence of measles virus RNA was detected in PBMC isolated from measles patients, and the production of the immunoreactive hemagglutinin protein was defective in these cells.

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