MEASLES INFECTION OF HUMAN MONONUCLEAR CELLS

I. Acute Infection of Peripheral Blood Lymphocytes and Monocytes*

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Although the pathophysiology of acute measles virus infection has been well described in the past (1), many of the immunologic sequelae of the illness have remained the subject of extensive investigation. The immune mechanisms underlying the observations that infants (2) and immunosuppressed children and adults (3) often succumb to a fatal form of this disease, that antibody (4) and protection from infection (2) can remain high for more than 60 yr in the absence of repeated antigenic stimulation, and that delayed cutaneous hypersensitivity (DCH) to tuberculoprotein is lost during acute measles infection (5) have all eluded definitive explanation. We have recently demonstrated that measles virus markedly inhibits the capacity of lymphocytes to respond to mitogenic stimulation in vitro (6) and speculated that direct infection of the lymphocyte by measles virus may be the cause of this suppression of immune responsiveness. Evidence that the virus replicates in lymphoid tissues producing characteristic cytopathic effect (CPE) or virus antigen (observed by immunofluorescence) was demonstrated in thymus (7,8), tonsils, lymph nodes, and spleen (7) of man or experimentally infected monkeys during acute infection. It also has been demonstrated that circulating lymphocytes taken during the acute stages of measles show evidence of measles virus infection when stimulated by phytohemagglutinin (PHA) (9).

The present series of experiments were performed in an attempt to define an invitro model to correlate with these clinical observations by determining the susceptibility of human mononuclear cells to measles virus infection. In addition to evaluating the effect of various mitogens on this susceptibility, the type of infection noted in mononuclear cells derived from infants and an adult without previous exposure to measles infection or immunization was compared to that seen in mononuclear cells from adults with historical or serologic evidence of prior measles infection.

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1 Abbreviations used in this paper: CMI, cell-mediated immunity; CON-A, concanavalin A; CPE, cytopathic effect; DCH, delayed cutaneous hypersensitivity; FA, fluorescent antibody; FCS, fetal calf serum; HI, hemagglutination inhibition; HSV, herpes simplex virus; PBL, peripheral blood lymphocytes; PBM, peripheral blood monocytes; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PFU, plaque-forming units; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SSPE, subacute sclerosing panencephalitis; VSV, vesicular stomatitis virus.

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Materials and Methods

Virus. The Edmonston wild (HEKs) measles virus strain passed seven additional times in a continuous African green monkey (Vero) cell line was used throughout the study. The viruses were propagated in cells maintained in Eagle’s minimal essential medium with 10% fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.). Suspensions of virus were harvested by collecting medium or homogenizing infected cell sheets at the time of maximal CPE. Virus pools contained $10^6-10^7$ plaque-forming units (PFU)/ml when assayed on Vero cell monolayers (10).

Human Blood Cell Cultures. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood of adults and from cord blood specimens obtained at delivery and separated by Ficoll-Hypaque gradient centrifugation (11). All adult donors, with the exception of one donor, had experienced prior measles infection as determined by serum hemagglutination inhibition (HI) titrations. The one adult donor with a negative measles history had no measles HI (<1:8) or neutralizing antibodies (<1:8). Cord blood specimens were obtained from healthy full-term neonates. Mononuclear cells were cultured in RPMI 1640 supplemented with 20% FCS, 100 U penicillin, and 100 μg streptomycin/ml. PBMC preparations contained 70-80% lymphocytes, 10-20% monocytes, and less than 10% polymorphonuclear leukocytes, as determined by cell morphology and latex particle phagocytosis. Peripheral blood lymphocyte (PBL) preparations were obtained by allowing monocytes to adhere to the surface of 32-oz glass tissue culture bottles overnight in the presence of 20% FCS. The nonadherent cells were poured off and these preparations contained fewer than 2% latex-ingesting monocytes.

Peripheral blood monocyte (PBM) preparations were obtained by placing $10^7$ PBMC in 2 ml of medium and allowing the monocytes to adhere to the surface of a 35 mm plastic petridish for 2 h at 37°C (12). The nonadherent cells were removed by washing the dish 4 times with medium. Preparations obtained using this method contained greater than 80% monocytes as determined by morphology and surface markers (12). In addition it was shown that the majority of adherent cells were monocytes by their ability to ingest latex particles. The adherent monocytes undergo morphological transformation to macrophages after being placed into tissue culture flasks which results in the presence of 20% FCS. The nonadherent cells were poured off and these preparations contained fewer than 2% latex-ingesting monocytes.

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Cell Counts and Viability. Total and differential cell counts were performed by the use of a crystal violet nuclear stain. Cell viability was assessed by 0.5% trypan blue exclusion. Latex particle phagocytosis was determined by incubating $1 \times 10^6$ cells in medium RPMI containing 20% FCS with 0.1 ml of 0.1% latex particles (1.1 μm diameter). After a 45 min incubation period the cells were washed 3 times in phosphate-buffered saline (PBS) and the percentage of cells containing ingested latex particles was determined by counting in a hemocytometer.

Mitogens. All mitogens were diluted in PBS, pH 7.4. Purified PHA (Burroughs Wellcome & Co., Inc., Research Triangle Park, N. C.) was used at a concentration of 1 μg/0.1 ml, concanavalin A (CON-A) (Calbiochem, San Diego, Calif.) at 12 μg/0.1 ml and pokeweed mitogen (PWM) (Grand Island Biological Corp., Grand Island, N. Y.) at 100 μg/0.1 ml. These concentrations of mitogen were found to produce maximum stimulation of lymphocytes as determined by $^3$H thymidine incorporation.

Infection of Blood Cell Cultures. PBMC and PBL were suspended in medium at a concentration of $1 \times 10^7$ml. 1 ml of cell suspension was added to each 16 x 125 tissue culture tube. Mitogen-stimulated cultures received 0.1 ml of PBS containing the appropriate amount of mitogen.

Control and PHA-stimulated cultures were incubated for 4 h at 37°C at which time the cells were sedimented, medium aspirated, and 0.2 ml of measles virus ($10^6-10^7$ PFU) was added to each tube. The tubes then were incubated for 1 h at 37°C in 5% CO₂ after which 0.8 ml of medium with or without 0.1 ml of PHA was added and the cultures were reincubated for an additional 72 h.

CON-A- and PWM-stimulated lymphocytes were incubated for 5 days, at which time the cells were sedimented and infected with measles virus in the same manner as for PHA-stimulated cultures. After infection, CON-A- and PWM-stimulated cells were incubated for an additional 24 h.

PBMC were stimulated in a one-way mixed leukocyte culture utilizing a mitomycin C-treated human lymphoblastoid cell line as the stimulus, as previously described (14). After 5 days of stimulation the cells were placed in culture tubes at a concentration of $1 \times 10^6$ml and infected with measles virus in the same manner as for PHA-stimulated cultures. After infection the cultures were incubated an additional 72 h.
PBM preparations were infected on the day of separation by adding 0.2 ml of virus (10^6–10^7 PFU) to each dish. The dishes were incubated for 1 h at 37°C in 5% CO₂, at which time 0.8 ml of medium was added to each dish and the cultures were incubated an additional 72 h. The adherent cells were removed by scraping with a rubber policeman and an infectious centers assay was performed. In addition, one experiment was performed where the monocyte preparations were placed in culture for a 2 wk period and infection was carried out on days 7 and 14, after morphologic transformation into macrophages had occurred.

**Supernate Titration.** Supernate harvests were titrated by the determination of either mean tissue culture infective dose values in microtitration plates (15) or by PFU in Petri dishes (10). Both techniques gave comparable results.

Briefly, the microtitration technique involved placing a suspension of Vero cells into each well of a microtitration plate (Falcon Microtest II; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and allowing the cells to incubate for 1 h at 37°C. 0.1 ml of serial 10-fold dilutions was then added to each of 4 or 8 replicate wells and the plate was allowed to incubate at 35°C. CPE was read at 5–10 days and 50% end points were determined by the method of Karber (16). PFU determinations were made by adding 0.2 ml of each virus dilution in duplicate to 35-mm plastic Petri dishes containing Vero cell monolayers. Cells were incubated for 1 h at 37°C and 3 ml of overlay medium were then added. After incubation at 35°C for 4 days, 1 ml of a 0.1% neutral red solution was added and the excess aspirated after 10 min. The dishes were allowed to incubate an additional 4–5 days before plaques were counted.

**Infectious Centers Assay.** Quantitation of the percent of cells containing infectious virus was performed using a modification of the technique of Jimenez et al. (17).

On the appropriate day after measles virus infection, lymphocyte and monocyte preparations were sedimented by centrifugation in 16 x 125 tissue culture tubes. Tissue culture medium was removed and the cells were incubated with 3 ml of a 1:30–1:50 dilution of equine antimeasles antibody which titered 1:4,096 by HI testing.

The cells were incubated for 1 h at 37°C and then washed 3 times with medium. Cell counts were adjusted to 5–100,000 cells/ml and 0.2 ml of each dilution was added to each of two 35-mm plastic petri dishes containing preformed Vero monolayers and allowed to incubate at 37°C for 1 h. 1 ml of overlay medium, was then gently added to the plates and the plates were allowed to remain immobile until the overlay had hardened. An additional 2 ml of overlay medium were added and the dishes handled as noted previously for PFU determinations. The number of plaques appearing divided by the number of cells initially added yielded the percentage of cells infected.

**Fluorescent Antibody (FA) Technique.** Intracellular measles antigen in acetone fixed lymphocytes was determined by the indirect FA technique as will be described in a later paper using hyperimmune monkey antisera.

Results

**Measles Virus Survival at 37°C.** Several experiments were performed where 0.2-ml samples of measles virus used during the study were added to tissue culture medium and incubated in tubes and microtiter plates at 37°C. Samples were removed at various times after inoculation and the titer of infectious measles virus was determined. Infectious virus was recoverable during the first several hours after incubation; however, after 24 h of incubation at 37°C no infectious measles virus remained.

**Infection of PBMC.** The results of infection of freshly isolated mononuclear cells from healthy adults in 10 experiments are summarized in Table I. The unstimulated PBMC and PBL preparations, which contained approximately 80 and 98% lymphocytes, respectively, yielded little virus 72 h after infection and

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1 Barry, D. W., J. L. Sullivan, S. J. Lucas, R. C. Dunlap, and P. Albrecht. 1975. Measles infection of human mononuclear cells. II. Acute and chronic infection of lymphoblastoid cell lines. Manuscript submitted for publication.
the cultures contained few giant cells and only 3–7% FA-positive cells. Few of the FA-positive cells (0.2%) yielded infectious virus as determined by the infectious centers assay and less than 10 PFU/ml of cell-free infectious virus were produced by these cells. The same cells, when stimulated with PHA, however, produced 625–1,600 PFU/ml of infectious virus in the supernatant fluid. The lymphocytes underwent extensive giant cell formation which comprised up to 15% of the total cells in some of the cultures, and 50–70% of the cells in culture showed evidence of measles antigen by FA. Although it would appear that only a small percentage of cells (7–8%) contained infectious virus as determined by infectious centers assay, these percentages are artifactually low since the majority of the infected cells originally in culture were included in giant cells, and a giant cell containing the nuclei of up to 50 infected lymphocytes would be considered a single infectious unit (Fig. 1).

Less than 1% of the monocytes were infected, as determined by characteristic CPE, FA, or infectious centers, although the few cells infected yielded 125 PFU/ml of virus in the supernatant fluid. The percentage of monocytes from the measles-nonimmune adult which were infected (0.4%) was in the range obtained with monocytes from measles-immune adults (0.01–0.5%). In order to determine if monocytes became more susceptible to infection after undergoing morphologic change to macrophages, monocyte preparations were infected at different time intervals after being placed in culture. Susceptibility to infection was measured by determining supernatant virus titers at various times after inoculation. In addition, monocyte preparations infected at days 0 and 14 were assayed for infectious centers 24 h after infection. Table II shows that monocyte preparations infected on days 0 and 7 and after 14 days in culture showed no striking difference in their ability to replicate measles virus as determined by supernatant virus titers and infectious centers assay. The number of cells containing infectious virus at day 14 after they had undergone morphological transformation into macrophages was 0.2% and not significantly different from the 0.4% level noted on day 0 when they were still monocytic in nature.

**Infection of Cord Blood Mononuclear Cells.** The results of infection of cord blood mononuclear cells are summarized in Table III and is compared with mononuclear cells from one measles-nonimmune and several measles-immune adult donors. Unstimulated cord blood mononuclear cells (primarily lympho-
FIG. 1. Multinucleated giant cell formed in measles virus-infected PHA-stimulated lymphocyte culture. Acridine orange stain. × 270.

cytes) were somewhat more susceptible to measles virus infection than adult PBMC, where only 0.01–0.5% of such cells showed evidence of infectious virus during the course of a number of experiments. Cord blood monocytes likewise were considerably more susceptible to measles virus infection than adult monocytes; 2–9% of the cord blood monocytes contained infectious virus. No significant differences were noted for monocytes or lymphocytes from the measles-nonimmune and measles-immune donors in comparing their susceptibility to measles virus infection and replication.

Effect of Mitogenic and Allogeneic Stimulation. The effect of different mito-
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TABLE II
Susceptibility of PBM to Measles Virus Infection

| No. of days in culture before infection | Cells infected at 24 h* | Supernate titer after infection |
|----------------------------------------|------------------------|-------------------------------|
|                                        | %                      | PFU/ml of supernate fluid     |
|                                        |                        | 24 h       | 48 h       | 72 h       |
| 0                                      | 0.4                    | 1,000     | 100        | 250        |
| 7                                      | ND‡                    | 125       | 630        | 79         |
| 14                                     | 0.2                    | 200       | 100        | 100        |

* As determined by infectious center assay.
‡ ND, not done.

TABLE III
Measles infection of Cord Blood Mononuclear Cells

| Subject                   | Cells infected* |
|---------------------------|-----------------|
|                           | PBMC            | PBMC + PHA | PBM      |
| Cord 1                    | 1.0             | 6.0        | ND‡      |
| Cord 2                    | 1.2             | 4.0        | 9.0      |
| Cord 3                    | 1.1             | 3.0        | 6.0      |
| Cord 4                    | ND              | ND         | 2.0      |
| Cord 5                    | ND              | ND         | 2.0      |
| Adult nonimmune           | 0.1             | 8.0        | 0.4      |
| Adult immune§             | 0.2 (0.01-0.5)* | 8.0 (4-16) | 0.2 (0.01-0.5) |

* As determined by infectious center assay.
‡ ND, not done.
§ 10 experiments.
* Mean (range).

gens and allogeneic stimulation on the susceptibility of lymphocytes to replicate measles virus is summarized in Table IV. PHA-stimulated cultures were infected 68 h before infectious centers assay while PWM-, CON-A-, and allogeneic-stimulated cells were infected for only 24 h before infectious centers assay. We have found that there is no appreciable difference in the number of infectious centers observed between 24 and 68 h after infection of PHA-stimulated lymphocytes (Sullivan, J. L., and D. W. Barry, unpublished observations).

The 3 mitogens induced an increased susceptibility to measles virus replication over that obtained with unstimulated cells. PHA (a potent T-cell stimulator) was associated with the highest percentage of infectious cells (4.0–16.0%), followed by CON-A (also a potent T-cell stimulator) (1.4–2.0%), while the percentage of cells stimulated by PWM (a T- and B-cell mitogen) which replicated virus was the lowest (0.8–0.9%). Allogeneic stimulation of lymphocytes using a mitomycin C-treated human lymphoblastoid B-cell line also was associated with a marked increase in cells replicating measles virus (4.0–5.0%) over that observed with nonstimulated cells (0.01–0.5%).
Discussion

Since the observation by von Pirquet in 1906 that delayed cutaneous hypersensitivity (DCH) to tuberculin was lost during measles infection (5), the immunologic and pathologic phenomena associated with infection by this myxovirus have been the subject of intensive investigation. The temporary loss of DCH to tuberculin has been repeatedly confirmed both during natural infection and after immunization with attenuated virus vaccine (18). Further evidence that the virus interferes with the normal function of the cell-mediated immune system may be found in the fact that the clinical course of untreated pulmonary tuberculosis worsens markedly during measles infection (19), while lipid nephrosis, which is responsive to both steroids and other immunosuppressants (20), improves during measles infection (21). Cell-mediated immunity (CMI) also has been shown to play an important role in recovery and protection from infection by measles virus as evidenced by the fact that children with hypogammaglobulinemia tend to experience a normal course of infection by this virus with lifelong protection from reinfection (22). Those with severe congenital or iatrogenic deficiencies of T-cell function, on the other hand, experience severe and often fatal infection with this virus (23). Data derived from the present study offer additional insight into the immunopathological mechanisms associated with measles virus infection in man.

It has been hypothesized that the loss of DCH during acute measles infection may be due to viral infection of immunocompetent cells (24) and measles virus antigen has been demonstrated in lymphoreticular organs of man and monkey during acute measles infection (7, 8). Osunkoya et al., (25) have induced measles-specific giant cells in PHA-stimulated cultures of lymphocytes obtained from children up to several days after the onset of rash. Our demonstration that stimulated lymphocytes can be infected with and replicate measles virus in vitro is supporting evidence that this may occur in vivo in the presence of background physiologic lymphocyte activation which occurs regularly in the thymus and lymph nodes (26). We have previously shown that measles virus-infected lymphocytes display an impaired response to in vitro PHA stimulation (6) and Mc-
Farland (27) has demonstrated that measles virus infection suppresses T-helper-cell function in vivo.

While previous studies have indicated that resting lymphocytes do not support viral replication (28), we observed low levels of measles virus replication in unstimulated peripheral blood mononuclear cells infected in vitro. This low level can be explained by minimal viral replication in monocytes; in addition, it was estimated that 0.06% of freshly isolated peripheral blood lymphocytes from adults are metabolically active as determined by [3H]thymidine incorporation (28). The increased frequency of measles virus replication in unstimulated mixtures of monocytes and lymphocytes from neonates (1%) as compared to similar mixtures obtained from adults (0.2%) can best be explained by the increased susceptibility of neonatal monocytes. Furthermore, a higher percentage of neonatal lymphocytes are metabolically active as determined by RNA and DNA synthesis (29).

Previous studies also have demonstrated that mitogen- and antigen-stimulated lymphocytes support the replication of many different viruses (28). Although measles virus has been isolated from leukocytes during acute infection (30) and shown to replicate in leukocytes cultured in vitro (31) the ability of the lymphocyte to support measles virus replication has not until now been well documented. We have shown that metabolic activation of lymphocytes by mitogens and allogeneic cells enables them to become infected and replicate measles virus. The ability to infect stimulated lymphocytes with measles virus is not affected by the immune status of the lymphocyte donor since lymphocytes from measles-immune donors supported viral replication in the range of that observed with lymphocytes from healthy neonates and a measles-nonimmune adult.

The cellular events responsible for the increased susceptibility of stimulated lymphocytes to viral infection and replication have not been elucidated. DNA synthesis does not become active in PHA-stimulated lymphocytes until the second day after stimulation (28). In the present study, stimulated lymphocytes were infected 4 h after the addition of PHA when the lymphocytes had actively begun to synthesize RNA and various proteins (28). The extent of viral infection and replication observed suggests that the cellular events which enable measles virus to infect and replicate in PHA-stimulated lymphocytes precede active DNA synthesis. An alternate explanation could be that while viral infection occurs early, as suggested by the absence of infectious virus in cell-free medium after 24 h at 37°C, measles virus replication does not occur until the second day when DNA synthesis has become active.

Kano et al., (32) demonstrated that activation of murine lymphocytes by mitogens other than PHA or by allogeneic stimulation resulted in an increase in the number of lymphocytes which replicate vesicular stomatitis virus (VSV). These authors used selective mitogen activation of murine T and B lymphocytes to demonstrate that VSV replicates in activated T lymphocytes. In the present study, human lymphocytes activated not only by PHA, but also by CON-A, PWM, or by allogeneic lymphocytes, were found to have an increased susceptibility to measles virus infection and replication. PHA and CON-A have been shown to primarily stimulate human T lymphocytes, while PWM stimulates
both human T and B lymphocytes, and the lymphocytes responding to allogeneic stimulation have been characterized as T lymphocytes (33, 34). The lack of a good selective mitogen for human B lymphocytes prevented us from determining the susceptibility of activated B lymphocytes to measles virus infection. Preliminary studies, however, using a rosetting technique to separate T and B lymphocytes have shown that human B lymphocytes obtained from tonsils are capable of being infected by measles virus (Barry D. W., and J. L. Sullivan, unpublished observations). These findings are in agreement with our data to be shown in a later paper that both human T- and B-lymphoblastoid-cell lines are capable of supporting measles virus replication.

The differences which we observed between cells stimulated by PHA and those stimulated by CON-A may be explained by the recent demonstration that PHA enhances fusion of cells infected with syncytial-forming viruses (35), while CON-A inhibits myxovirus release and virus-induced cell fusion (36). The low percentages of infection obtained with PWM are probably related to the low level of lymphocyte stimulation which we obtained with this agent. The number of blast cells observed in PWM-stimulated cultures was in the range of 20–30%, whereas PHA, CON-A, and allogeneic stimulation usually induced greater than 80% blast cells.

Previous studies demonstrated that many viruses can replicate in macrophages (37) and certain viruses, e.g. lactic dehydrogenase virus, replicate almost exclusively in this cell type (38). In the present study, monocytes from measles-immune adults and one measles-nonimmune adult were found to replicate measles virus at a very low level. Monocytes obtained from neonates, however, were capable of replicating measles virus in the range observed for mitogen-stimulated lymphocytes. The increased susceptibility of neonatal murine macrophages to infection and replication of herpes simplex virus (HSV) type 1 was demonstrated by Johnson (39), and such increased replication was related to HSV dissemination to the central nervous system in suckling mice (39). Hirsch et al. (40), have shown that the transfer of adult macrophages provides significant protection to suckling mice from HSV type I inoculated by intraperitoneal injection. It therefore has been suggested that the severe herpes virus infections which occur in human neonates may in part be due to impaired macrophage function (41). If the increased susceptibility of monocytes to measles virus infection observed in neonates persists through infancy the findings in the present study suggest that measles virus infection of children during infancy may result in increased viral replication in immature macrophages resulting in increased dissemination of the virus, particularly to the lungs and central nervous system. These findings may relate in part, to the increased severity of measles virus infections seen in children less than 1 yr of age (2, 42) and may also explain the high incidence of measles infection during the first 3 yr of life seen in patients who subsequently develop subacute sclerosing panencephalitis (SSPE) (43).

The demonstration that measles virus can infect and replicate in metabolically active lymphocytes resulting in inhibition of in vitro and in vivo immune responses may explain the depression of CMI associated with clinically acute measles virus infection in humans. More important, however, is the possible
long-term effects of such virus-lymphocyte interactions. Infection of lymphocytes in vivo by measles virus may allow for continued latent viral infection in the host with periodic activation providing antigenic stimulation for long-term immunity. In some instances, this activation may result in subacute or chronic infection as in SSPE or possibly multiple sclerosis (44).

Summary

A study of the susceptibility of human peripheral blood mononuclear cells to measles virus infection and replication is reported. Resting lymphocytes obtained from adults showed very low levels of infection and virus replication while lymphocytes activated by plant mitogens or allogeneic lymphocytes supported viral replication and underwent extensive giant cell formation. Unstimulated mononuclear cells obtained from the umbilical cord of healthy neonates were more susceptible to measles virus infection than those of adults; however, activated cord lymphocytes supported viral replication in the range observed with adult activated lymphocytes. Monocytes obtained from adults were relatively resistant to measles virus infection and replication while neonatal cord blood monocytes supported viral replication to the degree observed with activated lymphocytes. It is hypothesized that infection of activated lymphocytes may explain the depression of cell-mediated immunity seen during acute measles virus infection. The significance of the finding that neonatal monocytes are more susceptible to viral infection and replication than adult monocytes is discussed.

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