ACTIVATION OF MEASLES VIRUS FROM SILENTLY INFECTED HUMAN LYMPHOCYTES*

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A relation has been assumed between the pathogenesis of subacute sclerosing panencephalitis (SSPE)[1] and a preceding infection with measles virus that had been contracted some 10 yr before (1).

Although no formal proof exists that measles virus has remained present in individual children suffering from SSPE, this is generally believed to be the case. Thus it is not known where the measles virus resided during that period. It may be that, after a primary infection, resting lymphocytes or cells from the central nervous system harbor the virus. Indeed, it has been suggested that measles virus remains in the body of everyone because levels of antibodies against measles virus remain high during life, even in the absence of repeated antigenic stimulation from the environment (2). Measles virus has been isolated from human lymph nodes after recovery from the acute infection (3) and from lymph nodes of SSPE patients (4).

Previously, we have reported that measles virus inhibits the stimulation of lymphocytes in vitro (5). This effect is caused by the infection of lymphocytes rather than of monocytes (6).

Measles virus is known to be capable of infecting lymphocytes (7). However, it has been described that, for a productive infection, the lymphocytes must be stimulated (8, 9). In the present study the relation between lymphocyte stimulation and measles virus replication is further analyzed. As an explanation for the difference in susceptibility to virus infection between stimulated and nonstimulated lymphocytes, it has been suggested that measles virus is unable to enter nonstimulated cells, as though a necessary receptor is absent or masked (10). The present study shows that this is not so, but that the majority of nonstimulated lymphocytes do become infected. In the resulting infection, however, few of the apparently infected cells express demonstrable amounts of measles virus antigens. When the infected lymphocytes are subsequently stimulated with a mitogen, the measles virus is activated, resulting in the expression of measles virus antigen on most of the cells in the culture.

With some exceptions virus replication is not sustained in nonstimulated lymphocytes. Stimulation by mitogens makes lymphocytes susceptible to virus replication. Studies with persistently infected mice show that stimulation of lymphocytes with mitogens leads to activation of latent C-type viruses (11). Osunkoya et al. have described the activation of measles virus in lymphocytes obtained from children who

had recently recovered from a measles virus infection (12).

The present study may be regarded as an in vitro model of infection by measles virus in vivo.

Materials and Methods

Measles Virus. Edmonston B-strain measles virus was originally obtained from Dr. R. Brouwer, National Institute of Health, Bilthoven, The Netherlands. The virus was repeatedly passaged in Vero cells. Vero cells, known to be mycoplasma-free, were grown in medium 199 (Flow Laboratories, Inc., Rockville, Md.), supplemented with 5% calf serum (Flow Laboratories, Inc.). Stock virus was prepared in Vero cells using roller-bottle cultures that were infected at a multiplicity of infection (MOI) of 0.001. Supernate was harvested when maximal cytopathic effect was observed, which was usually around the 4th-day after inoculation. Supernates containing measles virus were clarified by low-speed centrifugation after which the virus suspension was stored at $-70°C$ in 1-ml aliquots. One sample was thawed and titrated after being frozen. Titers of infectious measles virus were between 10^7 and 5.10^8 tissue culture infective dose$_{50}$ per ml (TCID$_{50}$ per ml).

Titrations of measles virus were performed by adding serially twofold diluted samples of virus suspensions to eight replicate wells of flat-bottom microtest plates (Greiner M220-2gART, Greiner & Co., Inc., Nürtingen, W. Germany) in which Vero cell monolayers were grown.

All media and solutions contained 100 U/ml penicillin and 100 μg/ml streptomycin. All culture media contained additional glutamine. Cultures were performed in a humidified, 5% CO_2 atmosphere.

Infectious Center Assay. Infected mononuclear cells, which had been grown in medium containing fetal bovine serum (FBS), were treated with neutralizing antibodies against measles virus to remove extracellular virus. Infected cells, which had been grown to medium-containing pooled human serum, were counted and brought to the required concentration without any treatment. A known number of infected leukocytes was applied to Vero-cell monolayers in 60-mm Petri dishes. Noninfected leukocytes, treated in the same way, served as controls. 1-ml inocula were used and after a 2-h adsorption period excess inoculum was removed. The monolayers were overlaid with 0.6% agarose in medium 199, containing 10% FBS and additional L-glutamine. After 4 days the overlay was sucked off and cells were fixed with methanol and stained with Giemsa.

Preparation of Lymphocytes from Human Peripheral Blood. Peripheral blood lymphocytes from adult donors were obtained by centrifugation of diluted, defibrinated blood on a Ficoll-Isopaque cushion ($d = 1.077$). Cell suspensions collected from the interphase contained about 70-90% lymphocytes, 10-30% monocytes, and a few polymorphonuclear cells. Cell suspensions were washed four times with prewarmed medium containing 10% FBS before they were infected. Cells were counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). After cells had been stained with Türk solution differential counts were made microscopically.

Infection of Leukocytes with Measles Virus. Measles virus, diluted in RPMI-1640 medium with 25 mM Hepes as buffer (Grand Island Biological Co., Grand Island, N.Y.), was added to lymphocyte suspensions at a ratio of 2 TCID/50 per lymphocyte (MOI = 2). The virus was left with the nonstimulated lymphocytes for the times indicated in the various experiments.

Stimulation of Infected Lymphocytes. Infected lymphocytes were collected and washed twice with medium containing 10% heat-inactivated pooled human serum. The serum pool had a measles virus neutralization titer between 1:64 and 1:128. Between the two washings the infected cells were kept at 37°C for 1 h to neutralize any free measles virus. A third washing was included with 5% FBS supplemented medium. Immediately after the washings no infectious virus was found in the supernate. This means that $\approx 8.10^3$ viruses were removed (see the titer immediately before the washings as given in Table V). Cell numbers were determined in a Coulter counter. The viability of the cells, assayed by trypan blue exclusion, was always above 90%. Infected cells were subsequently cultured in medium containing human serum or FBS as indicated.

Depending on the kind of analysis to be made, different numbers of cells were cultured. For measurements of thymidine incorporation, 40,000 lymphocytes were cultured in a 150-μl vol in individual, round bottom wells of microtest plates (Greiner M220-24A), (13). Alternatively,

3.10^6 cells were cultured in 4 ml (Corning tubes, no. 25,200, Corning Glass Works, Science Products Div., Corning, N.Y.) or 0.3×10^6 cells in 1 ml (NUNC tubes, no. 1090). The medium was RPMI-1640 supplemented with either 15% of a selected batch of FBS or 20% pooled human serum. Phytohemagglutinin (PHA, Burroughs Wellcome & Co., Greenville, N.C.) was added to a final concentration of 50 μg/ml (microtest wells) or 25 μg/ml (1- or 4-ml tubes).

PHA stock solutions were diluted with RPMI-1640 medium buffered with Hepes. All culture media were buffered by hydrogen carbonate and contained 100 IU/ml penicillin, 100 μg/ml streptomycin, and additional L-glutamine. Cultures were performed in a humidified, 5% CO_2 atmosphere at 37°C.

Incorporation of [^{3}H]thymidine was measured after adding 20 μl of [^{3}H]thymidine (0.4 μCi, 200 mCi/mmol sp act) 72 h after the addition of PHA and 24 h before harvesting.

Details of tissue culture methods used in this laboratory can be found in reference 13.

Cytotoxicity Assay for the Detection of Measles Virus Antigens on Infected Cells (Target Cell Destruction Assay). Details of the assay are published elsewhere by Galama et al.[2] Fresh serum from a donor without previous transfusions or pregnancies was used as a source for both measles virus antibodies and complement. In some experiments serum was used from the same donor who had given blood for the preparation of the mononuclear cell suspension. No samples of serum showed a prozone effect when used in different dilutions. The assay was performed as follows. Infected and noninfected cells were labeled with ^{51}chromate by incubating the required number of cells with 100 μCi ^{51}Cr (Na$_2$^{51}CrO$_4$, Radiochemical Centre, Amersham, Great Britain, 100–300 mCi/mg, 1 mCi/ml) at 37°C for 60 min. After two washings, the suspension of labeled cells was brought to a density of $0,4–0,5.10^6$ cells/ml. From this cell suspension, 50 μl was incubated with 50 μl undiluted fresh serum. After an incubation at 37°C for 1 h, 900 μl of cold Earle's balanced salt solution (Flow Laboratories, Inc.) was added. The cells were then centrifuged at 1,800 rpm for 8 min. 0.5 ml was taken from the supernate (A) and counted separately from the remaining half (B). The percentage of released chromium was calculated as

$$\left(\frac{2A}{A + B} \right) \times 100.$$

Percentages of specific release given in the tables and figures have been corrected for maximal release (obtained by detergent lysis) and spontaneous release by the formula:

$$\text{specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}}.$$

Spontaneous release in 1 h seldom exceeded 15%.

Electron Microscopy. The required number of cells was pelleted and fixed in Karnovsky's fixative. The cell pellet was postfixed in a solution of 1% osmium tetroxide in phospahte, pH 7.5, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead hydroxide. At least 50 cells in each section were examined in a Philips EM 300 electron microscope (Philips Electronic Instruments, Inc., Mah Wah, N.J.) for the presence of virus particles.

Results

Proliferating Capacity of Infected Lymphocyte Suspensions. To test whether lymphocytes become infected with measles virus we incubated nonstimulated lymphocytes for 96 h with measles virus, added at a multiplicity of 2 TCID$_{50}$ per cell (MOI of 2). After the incubation period the cells were extensively washed with medium containing antibodies against measles virus. The cells were then resuspended in culture medium containing measles virus antibodies and stimulated with PHA.

[2] J. M. D. Galama et al. 1978. Lymphocyte-mediated cytotoxicity to cells infected with measles virus. I. Use of in vitro infected leukocytes as autologous target cells; absence of virus-specific cytotoxic lymphocytes. *Cell Immunol.* **38:** 365.

TABLE I

Stimulation with PHA of Mixtures of Measles Virus-Infected Lymphocytes and Noninfected Lymphocytes

Measles virus-infected cells*‡	Noninfected cells*	Inhibition§
		%
+	−	82
+	+	−35 ‖

* 40,000 infected and/or 40,000 noninfected cells were cultured together in the presence of pooled human serum.
‡ Infection with measles virus was performed 96 h previously at MOI = 2.
§ Mean result of four experiments is given.
‖ The negative percentage inhibition means that, most likely owing to a feeder effect, the cpm in the mixtures were higher as compared with the cpm in noninfected cells alone.

TABLE II

Thymidine Incorporation by PHA-Stimulated Lymphocytes Incubated Previously for 96 h with Measles Virus

	Infected cells*	Noninfected control	Inhibition‡
	cpm 10^{-3}	cpm 10^{-3}	%
Stimulated cells cultured in pooled human serum	4.5 ± 0.7	39.9 ± 4.3	89
Stimulated cells cultured in FBS	0.8 ± 0.2	32.5 ± 5.1	98

* Mononuclear cell suspensions were infected with measles virus at MOI = 2. After incubation for 96 h in medium supplemented with 15% FBS, free virus was removed by washing with measles antiserum as described in Materials and Methods. 40,000 cells were added to triplicate wells of microtest tissue-culture plates and PHA was added. One typical experiment out of a series is given. Identical results were observed as control values in all other experiments.
‡ % Inhibition is calculated as:

$$100 - \left(\frac{\text{cpm culture + virus}}{\text{cpm culture without virus}} \times 100 \right).$$

In a previous paper (5) we reported that (*a*) measles virus inhibits DNA synthesis in PHA-stimulated lymphocytes and (*b*) this inhibition can be prevented entirely if antibodies against measles virus are present. The antibodies block the release of measles virus and prevent spreading of the measles virus to other cells (The experiments which demonstrate this point are summarized in Table I; extensive data can be found in reference 6).

The results of measuring thymidine incorporation, after PHA stimulation of the lymphocytes, that were preincubated with measles virus, are given in Table II. The DNA synthesis was almost completely inhibited. Because this cannot be due to released soluble factors (6), we took this result as an indication that the majority of the lymphocytes had been infected with measles virus.

Next, experiments were performed to investigate the influence of the length of time during which lymphocytes and measles virus were incubated together. Table III shows that it took about 96 h to infect the majority of the cells, based on the criterion of the remaining DNA synthesis in response to PHA stimulation. Table III also shows that, if infected cells were cultured with PHA in medium containing FBS instead of pooled

TABLE III

Inhibition of Thymidine Incorporation by PHA-Stimulated Lymphocytes Previously Incubated with Measles Virus for Various Times

Incubation period of non-stimulated lymphocytes with measles virus‡	Inhibition of thymidine incorporation*	
	PHA stimulation in pooled human serum	PHA stimulation in FBS
h	%	
24	4	91
48	17	90
72	36	93
96	77	97
120	88	NT§
192	90	NT
360	90	NT

* Calculated as described in the legend to Table I.
‡ Incubation of cells with measles virus in medium containing 15% FBS.
§ NT, not tested.

TABLE IV

Lysis by Fresh Human Serum of Lymphocytes Infected with Measles Virus

	Specific release of ^{51}Cr*		
	Lymphocytes‡ + measles virus§ + PHA ‖	Lymphocytes + measles virus without PHA	Lymphocytes without measles virus + PHA
	%		
Fresh human serum	92	3	3
Heat-inactivated serum (56°C, 1 h)	3	0	1

* Mean result of two cultures in one experiment.
‡ Lymphocytes were incubated for 72 h.
§ Measles virus added at t = 0, MOI of 2.
‖ PHA added at t = 0; culture performed in medium containing FBS.

human serum, a previous 24-h incubation with measles virus was sufficient to allow the infection to proceed rapidly in the presence of PHA. We have no adequate explanation for the finding that 96 h incubation of measles virus and lymphocytes is required to lead to an almost complete inhibition of DNA synthesis during a further culture period with PHA in the presence of antibodies against measles virus. The long incubation may also be needed because it might take 96 h to infect lymphocytes to such an extent that the proliferating capacity is impaired.

After the majority of the cells had become infected on the 4th-day after infection, the cells remained alive and infected for at least 15 days. Evidently no extensive cell lysis of lymphocytes infected with measles virus took place.

The incubation of lymphocytes with heat-inactivated measles virus had no influence on their ability to respond to PHA.

Expression of Measles Virus Antigens on the Cell Surface. Lymphocytes that had been incubated with measles virus during 96 h were not susceptible to lysis in the presence of antibodies against measles virus and complement, whereas the same cells, if stimulated with PHA, added together with measles virus, could easily be destroyed by antibodies and complement (Table IV). Subsequently, we investigated whether
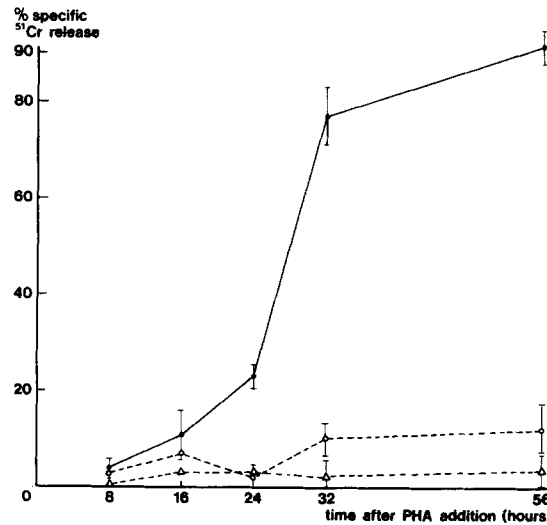
Fig. 1.   Expression of measles virus cell surface antigens in relation to time after the addition of PHA to silently infected lymphocytes. Abscissa, time of PHA addition after the 96 h preincubation of lymphocytes with measles virus. Ordinate, percent specific release of ^{51}Cr, corrected for maximal and spontaneous release as described under Materials and Methods. The data shown are mean results of four experiments ± 1 SD. A- - -A, cultures of noninfected lymphocytes, with PHA; O- - -O, cultures of measles-infected lymphocytes, without PHA; ●——●, cultures of measles-infected lymphocytes, with PHA.

TABLE V

Measles Virus Titer in Supernate of Nonstimulated Lymphocytes

Time of incubation	Measles virus titer in the presence of cells*	Measles virus titer in the absence of cells*
h		
24	4.2	3.7
48	3.6	1.8
72	3.4	<0.7
96	3.3	<0.7

* 6.10^6 Titer expressed as ^{10}log TCID$_{50}$/ml were added to 3.10^6 lymphocytes in 4 ml medium.

cells, that had been incubated with measles virus during 96 h in medium with FBS and after that with heat-inactivated human serum to remove free virus, and then stimulated with PHA, could be killed by antibodies against measles virus and complement. Since culturing the cells with PHA in the presence of antibodies against measles virus would presumably interfere with the expression of measles virus antigen, these cultures were performed with FBS.

The results are presented as a time curve in Fig. 1. Within 32 h after the addition of PHA the majority of the cells became susceptible to lysis by antibodies against measles virus and complement. Induction of measles virus antigen within 48 h was also observed when PHA was added 8 or 15 days after infection of the lymphocytes with measles virus (data not shown).

Induction of Release of Infectious Virus.   Sullivan and co-workers (8) observed that 10^7 infectious measles virus particles disappeared in tissue culture medium within 24 h at 37°C. As shown in Table V, we observed a somewhat longer half-life, observing that

TABLE VI

Measles Virus Titer in Supernate of Lymphocytes Which Were Infected before Stimulation by PHA

Time of incubation of nonstimulated lymphocytes with measles virus	Treatment	Measles virus titer* (^{10}log $TCID_{50}$/ml)	
h		+PHA	−PHA
96	Cells washed with 5% FBS before PHA addition	5.4	2.0
96	Cells washed with 10% pooled human serum before PHA addition	6.0	1.7
192	Cells washed with 5% FBS before PHA addition	4.3	2.0
192	Cells washed with 10% pooled human serum before PHA addition	4.9	1.4

* Supernatant samples were taken 72 h after PHA addition or after sham treatment.

TABLE VII

Measles Virus Titer in Supernate of Lymphocytes Which Were Infected before Stimulation by PHA

Time after the addition of PHA*	Mean measles virus titer‡ (n = 4) (^{10}log $TCID_{50}$/ml)
h	
6	<2
18	<2
24	<2
48	4.8
72	5.6
96	5.6

* PHA added at 96 h after infection. Before addition of PHA cells were treated with antibodies to measles virus as described.
‡ Mean titer of four determinations obtained in two experiments; supernatant samples for titration taken 72 h after the addition of PHA.

it took more than 48 h to inactivate 6.10^6 viruses. In the same Table V, the measles virus titer in the supernate of nonstimulated lymphocytes is given. Since this titer remains higher in comparison with virus incubated in the absence of cells, it is likely that some virus production is going on in the cell pellet. Thus, measles virus replication took place to only a low extent in nonstimulated cultures (Table VI).

When PHA was added to lymphocytes that had previously been incubated with measles virus for 96 h and treated with antiserum, the titer of infectious virus in the supernate increased rapidly (Table VII). Between 24 and 48 h the titer in the supernatant medium jumped from near zero to a plateau value. This time curve was similar to that obtained with regard to the expression of measles virus antigen.

Analysis of the Number of Infected Cells by the Infectious Center Assay. When a known number of infected nonstimulated lymphocytes was applied to a Vero cell monolayer and subsequently immobilized by an agarose overlay, about 1 in 5,000, or less, cells gave rise to a plaque. The addition of PHA, 96 h after infection with measles virus, resulted in an increase in the number of virus-producing cells. The PHA-stimulated lymphocytes were cultured in pooled human serum containing antibodies against measles virus. Cells were collected at various times after PHA addition and applied

TABLE VIII

Infectious Center Assay of Stimulated, Silently Infected Lymphocytes

Time after PHA addition*	Number of virus-producing lymphocytes after stimulation of silently infected cells Stimulation in pooled human serum‡
h	
4	0.3
8	0.3
16	0.8
32	3
Without PHA	0.1§

* PHA added 96 h after infection. Before addition of PHA, cells were treated with antibodies to measles virus as described.

‡ Number of virus-producing cells/1,000 lymphocytes.

§ Nonstimulated cells were after the 96-h preincubation cultured for 32 h in pooled human serum.

in known numbers to Vero cell monolayers. Table VIII shows that, in a cell suspension, taken 32 h after the addition of PHA, one in 300 cells produced virus.

Without stimulation only about 1 in 5,000 cells produced virus, whereas 32 h after PHA stimulation this proportion was found to be around 1 in 300. These results should, however, only be interpreted in a qualitative way. Recently, we observed that the infectious center assay underscored the actual number of virus-producing cells. This was suspected to be, among other factors, the result of the presence of antibodies against measles virus in the culture medium. On the other hand, the occurrence of giant cells in the infected, stimulated cultures (1, 8) might result in lower numbers of virus-producing cells if these are counted in a Coulter counter on the basis of the number of nuclei they contain. Moreover, the results of the infectious center assay varied from experiment to experiment, but never varied much between different cell suspensions used in one experiment.

Electron Microscopy of Infected Lymphocytes. Infected lymphocytes were fixed and prepared for electron microscopy. In cells that had been incubated with measles virus without a mitogen, no virus structures were found. Only healthy, intact, completely normal mononuclear cells were seen. However, cells to which PHA had been added 96 h after virus infection, showed evidence of heavy virus infection when cultured with FBS or pooled human serum. For example, previously infected lymphocytes which were cultured with PHA in the presence of antibodies against measles virus showed, when looked at 72 h after the addition of PHA, virus particles in 159 of 221 sections. Generally, in more than half of the cells, studied 48 h or more after the addition of PHA, large arrays of measles virus nucleocapside structures could be seen (Fig. 2). This implies that most cells had been infected, because only a section of each cell can be investigated. We had the impression that the cells which were cultured in FBS showed more virus structures at an earlier time after the addition of PHA; at 72 h, however, no difference was observed.

Discussion

The model system described here shows that measles virus can enter a cell and remain there without presenting visual evidence of its presence. This study, confined to human lymphocytes, demonstrates that nonstimulated lymphocytes can become
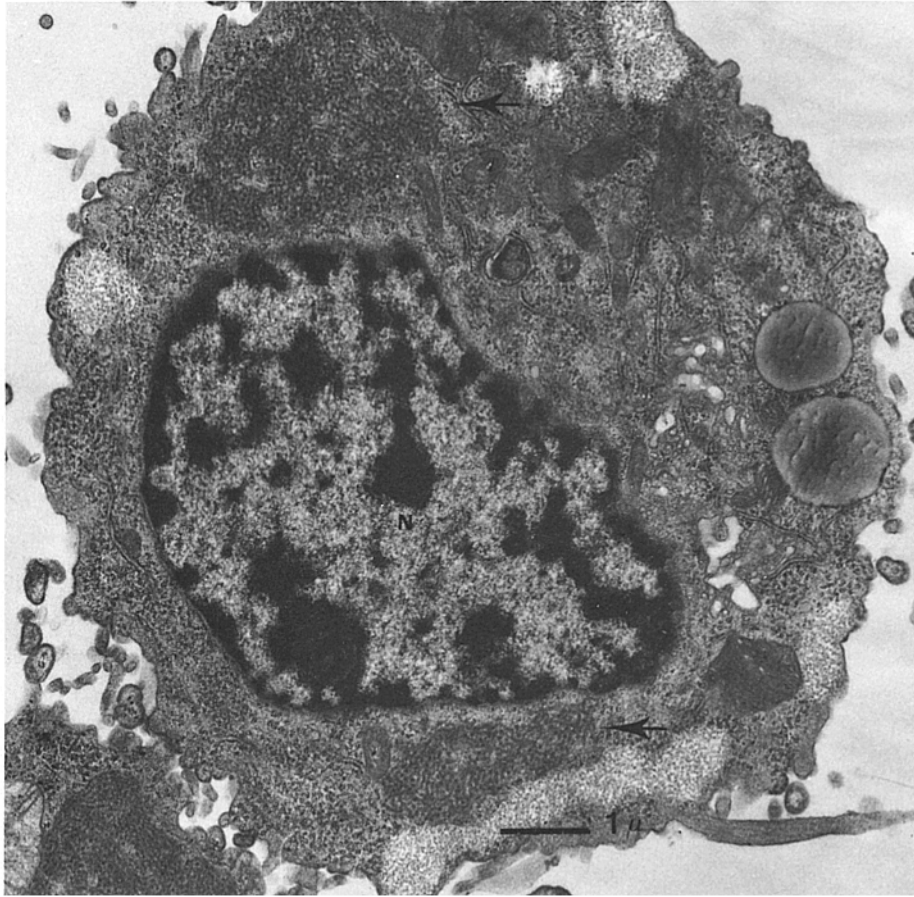
Fig. 2.   Lymphocyte infected with measles virus, 48 h after stimulation with PHA in medium with FBS. Cells infected 96 h before the addition of PHA.

infected with measles virus. This means that the well-known block for virus replication in nonstimulated lymphocytes is localized after penetration of the virus into the cell. Recently, it was observed that a similar situation is found in nonstimulated mouse lymphocytes, infected with vesicular stomatitis virus (14).

We propose to designate such an infection as silent to avoid confusion with latency, a term generally used for the situation where virus genomes are integrated into host cell genetic material. The silent infection, described in this study, differs from the well-known carrier fibroblasts persistently infected with measles virus (15, 16) in that measles virus antigens are expressed on the surface of the latter and not of the former. A persistent infection by measles virus with a concomitant low production of virus and extensive antigen expression in lymphoid cell lines has recently been reported (17). It seems likely that persistant infections with virus-specific antigens, being expressed on the majority of the cells, can be observed in cultures of other, permissive cells with an active metabolism. This is in agreement with our observation that stimulation of the silently infected cells with a mitogen leads to activation of the measles virus. Apparently, the activation of the virus does not require the induction

of DNA synthesis since the incorporation of thymidine is virtually absent in the infected cells and, moreover, mitomycin C does not interfere with the activation of measles virus.[3]

It is difficult to indicate to what extent the suspension of lymphocytes is infected after the 96-h incubation period before mitogen stimulation. Two sets of experiments could be performed under conditions that exclude spreading of the measles virus to other cells after the addition of the mitogen. These conditions imply the presence of antibodies against measles virus in the tissue culture medium during the period in which PHA is present (5, 6). The experiments we refer to involved the measurement of DNA synthesis and of infectious centers (virus-producing cells). We have evidence that the latter assay underscores the number of infected cells actually present, and therefore does not give quantitative information. Thus we are left with the remaining DNA synthesis in infected cells as a more or less quantitative indication of the number of infected cells immediately before the addition of the mitogen. It is only on this consideration that we simplify the notion extent of inhibition of thymidine incorporation to number of infected cells. Thymidine incorporation of nonstimulated lymphocytes (Table III) is inhibited up to 90% after incubation for 96–120 h. When a constant number of cells in different ratios of infected- to noninfected lymphocytes was cultured in medium supplemented with serum that contained antibodies against measles virus, a linear relation was found between the extent of inhibition and the number of infected lymphocytes present. This means that the percentage of inhibition may indeed be taken as reflecting the approximate number of infected cells. When nonstimulated, virus-infected lymphocytes are stimulated with concanavalin A or irradiated allogeneic cells in the presence of measles virus antibodies, the extent of inhibition of DNA synthesis is somewhat smaller in comparison with the inhibition observed after PHA stimulation, i.e. about 70% instead of 90% (6). No adequate explanation for this difference can be given, but the finding implies that we should not too strictly compare inhibition with infection.

Other assays, e.g. infectious center assay, release of infectious virus, electron microscopy, and induction of measles virus cell surface antigens, demonstrate that the activation process occurs between 16 and 30 h after the addition of the mitogen. Portner and Bussell showed that, after infection of human amnion cells by measles virus, it took more than 18 h to detect the first progeny virus (18). We could not find any virus 24 h after stimulation. If this is a real delay it might be explained by the fact that in the infected lymphocyte the mitogen-induced metabolic activities must first be initiated. On the other hand, we did not exclude adsorption of progeny virus to adjacent cells. Still, these observations made it unlikely that, during the first 30-h after the addition of PHA, many secondary infections would have taken place. Obviously, a minor contribution of virus spreading cannot be excluded.

The precise role of antibodies against measles virus with regard to the expression of measles virus needs further study. Silently infected cells, that were stimulated by PHA in the presence of measles virus antibodies, could not be lysed. This was to be expected since it is well known that antibodies against measles virus can strip infected cells, thereby rendering them nonsensitive to lysis by antibodies and complement. Our results might confirm Joseph and Oldstone's observation (19) that cell surface antigens are perhaps not the only markers influenced by measles virus antibodies. From the

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[3] C. J. Lucas et al. Manuscript in preparation.

electron microscopy studies presented here it seems that intracellular production of measles virus may be influenced by the presence of measles virus antibodies in the tissue culture medium. However, electron microscopy is a rather insensitive method and small areas of virus activity in a few cells may easily be missed.

It is noteworthy that lymphocytes require incubation for 96 h with measles virus to infect the majority of the cells. The number of infected cells could not be raised by increasing the MOI from 2 to 8 (data not shown). The experiments (Table III) show that the number of infected cells increased during this period. This might mean that secondary infections took place during this period. If this be so, it is not clear how, after the original infection (involving only a few cells), the infection was spread in the absence of overt virus production. The possibility cannot be excluded that stimulating factors in the FBS played a role in this spreading, although when measles virus was added to lymphocytes that had been incubated in FBS-containing medium for 4 days, a similar infection curve was obtained as is shown in Table III. When infected cells are subcultured in human serum without a mitogen they remain infected for at least 4 days (data not shown). Previous studies, as well as experiments reported by Gallagher and Flanagan, showed that infection by measles virus in the presence of PHA or the infection of lymphoblastoid cell lines involved only few cells initially (5, 20).

It is not clear why virus replication is absent in nonstimulated lymphocytes. Nowakowski et al. reported that the block for the replication of vesicular stomatitis virus in a lymphoid cell line is associated with a limited production of 42 S virion RNA (21). Stimulation of lymphocytes by a mitogen induces a whole series of metabolic processes. It would be guessing to trace the specific one(s) that are required for measles virus replication. Recent experiments have shown that the induction of measles virus antigens on the surface of the stimulated, infected lymphocytes can be inhibited by a high concentration of adenosine and by azaserine.[3] This suggests that at least the de Novo synthesis of purines is required for measles virus replication. It remains to be established whether this is also the metabolic event that is lacking in virus-infected nonstimulated lymphocytes.

The stock virus used for the infection of lymphocytes was prepared by infecting Vero cells at an MOI of 0.001, which was the value at which Rima et al. observed the least amount of defective interfering (DI) particles (22). Hence it is unlikely that the silent infection is initiated by contaminating DI particles in the stock virus. Future work will aim at a study of the nature of the viruses released after activation of the lymphocytes. Perhaps DI particles and temperature-sensitive mutants arise during the virus replication in activated lymphocytes.

Cytomegalo virus may also be able to infect nonstimulated lymphocytes (23, 24). The model presented in this study, however, is the first to cover details of virus infection of nonstimulated cells. The results show that a virus-infected lymphocyte, if not stimulated, can harbor the virus without being recognizable for immunological attack.

Summary

Lymphocytes were incubated with measles virus for 4 days in the absence of a lymphocyte stimulating agent. Such nonstimulated lymphocytes, infected with measles virus, did not express the virus antigens that are detectable by cytotoxic antibodies.

Approximately 1 out of 5,000, or even fewer, of such lymphocytes produced virus as demonstrated by the infectious center assay; in the supernate only 10–100 infectious viruses per milliliter were detected. No virus structures could be observed by means of an electron microscope.

However, such lymphocytes showed no reaction to phytohemagglutinin (PHA) in terms of DNA synthesis in a subsequent culture in the presence of antibodies against measles virus to prevent spreading of the infection to other cells.

Although stimulation by PHA did not result in a significant increase in [^{3}H]-thymidine incorporation, measles virus was activated; 32 h after the addition of PHA nearly 80% of the cells were killed by measles virus antibodies and complement. The number of virus-producing cells increased to approximately 1 in 300 or more, and at 72 h the virus titer in the supernate had risen to 10^6 infectious particles per ml. This reactivation of measles virus was still obtained when PHA was added as late as 8 or more days after the initial infection.

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