Measurement of Virus Antigens on the Surfaces of HeLa Cells Persistently Infected with Wild Type and Vaccine Strains of Measles Virus by Radioimmune Assay

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SUMMARY

Persistent states of measles virus infection have been established in HeLa cells by using Edmonston strain virus and two types of measles virus vaccine (M-VAC and Schwarz). The absolute amount of surface viral antigens expressed on these cells infected separately with the three viruses has been assessed by a newly developed method which employs \[^{125}\text{I}\]-labelled Fab fragments of immunoglobulin G (IgG) from immune human sera. This method was used to determine the level of viral antigenic expression on acutely infected HeLa cells harvested at a time when 95 to 100% of cells could be lysed by antiviral antibody and complement. From our data, more than $1 \times 10^6$ antibody molecules must bind to each cell infected with measles virus before complement dependent lysis can occur in a homologous test system. Persistently infected cells bind 2 to 3 times less antibody than acutely infected cells and correspondingly exhibit less susceptibility to humorally-mediated immune lysis.

INTRODUCTION

During the past decade, a number of studies (Norrby, 1967; Minagawa, 1971; Raine et al., 1971; Feldman et al. 1972) have corroborated Rustigian's (Rustigian, 1966) original observation that persistent states of measles virus infection can be established in culture systems acutely infected with wild-type measles virus. Other in vitro studies have indicated that comparable results may be obtained with vaccine strains of measles virus (Webb, Illavia & Laurence, 1971; Knight, Duff & Rapp, 1972; Bather et al. 1973). From these collected accounts, it is clear that persistent states of measles virus infection can evolve even in environments devoid of immunological pressures. Recently, attention has been refocused on these test systems in the hope of gaining further insight into the pathogenesis of human subacute sclerosing panencephalitis (SSPE) — a progressive degenerative disease whose link with measles virus is now firmly established (ter Meulen, Katz & Müller, 1972).

To our knowledge, no group has established persistent infections with wild-type virus and measles virus vaccines in cells derived from a single tissue culture line. This experimental design permits precise comparisons to be made between cells persistently infected with either wild type or vaccines of measles virus. For example, we demonstrate that HeLa cells persistently infected with vaccine strains of measles virus differ quantitatively from HeLa cells persistently infected with wild-type measles virus with respect to both surface viral
antigenic expression and immunolytic susceptibility. Significant differences in these parameters were also evident between persistently and acutely infected HeLa cells.

METHODS

**Virus.** Wild type Edmonston strain measles virus was obtained from the American Type Culture Collection, Rockville, Maryland. Two attenuated strains of measles virus (M-VAC vaccine, American Cyanamid Co., Lederle Laboratories Div., Pearl River, N.Y.; Lirugen Schwarz strain vaccine, Dow Chemical Co., Midland, Michigan) were obtained from the Scripps Clinic Pharmacy. Pools of each virus were prepared as outlined elsewhere (Joseph, Cooper & Oldstone, 1975).

**Persistently infected cells.** HeLa cells were obtained from Microbiological Associates, Los Angeles, Calif. These cells were examined for mycoplasma contamination; none was evident by culture or by ultrastructural assessment. Separate groups of cells (1 x 10⁷) were each infected with a different one of the three virus stocks at a multiplicity of less than 1.0. Cells were grown as monolayers in 75 cm² Falcon flasks (Falcon Plastics, Div. of Bioquest, Oxnard, Calif.) with Eagle's minimal essential medium supplemented with 10% heat-inactivated foetal calf serum, 1% glutamine and antibiotics (growth medium). Flasks were incubated at 37 °C in 5% CO₂. After infection, cultures were divided as often as necessary to maintain a subconfluent condition. During the first 2 weeks of infection, cultures were refed on a daily basis; thereafter, cultures were refed at least twice weekly. By the fourteenth day of infection, most of the cells in each culture were degenerate. Surviving cells progressively increased in number and by the sixth week of infection had become sufficiently expanded to permit serial passaging. Cells were grown for an additional 8 weeks before experimental assessment. During the week immediately preceding experimental assay, each persistently infected cell line was refed on a daily basis so as to maximize viability. One HeLa cell line persistently infected with various strains of measles virus was used in these experiments.

**Acutely infected cells.** HeLa cells (1 x 10⁷) were infected with Edmonston strain measles virus at a multiplicity of 1.0. Virus adsorption was carried out for 4 h at 37 °C in a closed vessel affixed to a continuous inverter. Cells were then plated into 75 cm² Falcon flasks containing fresh growth medium. Flasks were incubated under the conditions described above with daily refeeding. Cells were harvested for experimental analysis on the third to fourth days of infection (i.e., when all adherent cells exhibited some cytopathic abnormality by phase microscopy).

**Harvesting cells for experimental assay.** Monolayers infected with measles virus were washed repeatedly in situ with buffered saline so as to remove poorly adherent cells and the majority of free virus. Adherent cells were then covered with 0.05% trypsin-EDTA solution (Grand Island Biological Co., Grand Island, N.Y.) and gently agitated at room temperature for 1 to 2 min until completely suspended. Cells were then washed repeatedly under conditions known to preserve viability (Joseph et al. 1975). All of the preparations used in this study exhibited a starting viability of 95% or greater as judged by trypan blue dye exclusion.

**Immunofluorescence and micro-cytotoxicity assays.** A detailed description of the techniques employed is presented elsewhere (Joseph & Oldstone, 1974; Joseph et al. 1975).

Immunofluorescence was used to assess surface measles virus antigens on viable cell suspensions stained by human anti-measles IgG tagged with fluorescein isothiocyanate (FITC).

Each test mixture in the cytotoxicity assay consisted of 8 µl of freshly thawed, measles
Surface expression of measles virus antigens

immune human serum plus 1 µl of suspended cells at an approximate concentration of 3 x 10^6 cells/ml. Control reactions employed heat-inactivated (56 °C, 30 min) immune serum from the same human source.

Radiolabel assay: measurement of measles virus antigens on cell surfaces. IgG fractions were isolated (Fahey, 1967) from the serum of an adult human with a childhood history of rubeola and from the serum of a patient with SSPE. The haemagglutination inhibition titres of these sera were 1:64 and > 1:1000, respectively. Fab fragments (Fab-adult and Fab-SSPE) were then prepared from each IgG fraction by papain digestion and chromatography (Franklin, 1960). It was necessary to use Fab fragments rather than whole IgG molecules because HeLa cells have surface Fc receptors (A. Theofilopoulos & B. S. Joseph, unpublished observations). Fab isolates were labelled with [125I] by the chloramine-T method (McConahey & Dixon, 1966) so as to obtain a sp. act. of at least 0.5 µCi/µg. Approx. 0.7 % of Fab-adult was specific for measles virus as compared with 12 % for the Fab-SSPE isolate. Radio-labeled Fab preparations were deaggregated by centrifuging at 100,000 g for 30 min; unlabelled, ‘cold’ Fab isolates were individually deaggregated in a similar manner.

Cells of each type (i.e., acute measles, Edmonston persistent, M-VAC persistent, Schwarz persistent, and uninfected HeLa cells) were placed in individual 13 x 100 mm glass tubes. A spectrum of four different cell concentrations was employed for each line: 5 x 10^4, 1 x 10^5, 5 x 10^6, and 1 x 10^8 pelleted cells per tube. Experiments with Fab-adult and with Fab-SSPE were performed on separate occasions. In each instance unlabelled Fab was mixed with radiolabelled Fab in a ratio of 10:1. With Fab-SSPE, 25 µg (200 µl) were added to each cell sample; with Fab-adult, 200 µg (200 µl) were added per cell sample. Fab-cell mixtures were incubated for 45 min at room temperature with intermittent shaking. Cells were then washed 5 times with 7 ml cold (4 °C) growth medium.

The residual radioactivity remaining in a cell-associated state in each sample was then determined by using a Baird atomic gamma counter. Counts obtained for uninfected HeLa cells were subtracted from the counts obtained for each of the infected samples. By taking account of the dilution with unlabelled Fab, the sp. act. of the Fab mixtures employed, the mol. wt. of Fab (viz., 52,000), the adjusted number of counts per sample and the number of cells per sample, we could determine the number of Fab fragments binding specifically per cell in each sample by the following formula:

\[
\frac{(6.023 \times 10^{20}) \times \mu g \text{ Fab fixed on the cells}}{\text{number of cells} \times \text{mol. wt. of Fab}} \times 10^{-6}.
\]

Cells from each population studied by radiolabelling were also evaluated by immunofluorescence and micro-cytotoxicity assays.

RESULTS

HeLa cells could be persistently infected with two attenuated strains of measles virus, as well as with wild type Edmonston strain measles virus. The appearance of these three lines was indistinguishable by phase microscopic criteria. Some syncytial and giant cell formation was evident in all cultures, with the majority of cells in each culture appearing normal. Confluency could be attained for each persistently infected line and cell health maximized by a regime of frequent refeeding and by dividing cultures at least once weekly. Studies not detailed here (B. S. Joseph, P. W. Lamper & M. B. A. Oldstone, unpublished observations) indicate that these three cell lines exhibit an essentially comparable ultrastructural appearance.
Fig. 1. Phase photomicrographs of (a) uninfected HeLa cells, (b) HeLa cells persistently infected with Schwarz strain measles virus and (c) HeLa cells acutely infected with Edmonston strain measles virus. Photomicrographs (d) to (f) depict immunofluorescence staining reactions for surface measles virus antigens in each of the above cell lines, respectively. Standardized conditions of illumination and exposure were employed for illustrations (d) to (f).
Surface expression of measles virus antigens

In Fig. 1, phase and fluorescence photomicrographs are presented for uninfected HeLa cells, for HeLa cells persistently infected with Schwarz strain virus and for HeLa cells acutely infected with wild-type measles virus. Approximately comparable staining reactions were obtained for cells persistently infected with Edmonston and M-VAC strains as that illustrated for cells persistently infected with Schwarz strain virus. Uninfected HeLa cells...
Table 1. Immunofluorescence and cytotoxicity studies on cells used for radiolabelling assays*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IF⁺ (%)</td>
<td>Lysis§ (%)</td>
</tr>
<tr>
<td>(1) Acute Edmonston</td>
<td>100 0.99</td>
<td>100 0.95</td>
</tr>
<tr>
<td>(2) Edmonston P/M</td>
<td>95 0.70</td>
<td>95 0.75</td>
</tr>
<tr>
<td>(3) M-VAC P/M</td>
<td>95 0.65</td>
<td>75 0.35</td>
</tr>
<tr>
<td>(4) Schwarz P/M</td>
<td>90 0.35</td>
<td>65 0.25</td>
</tr>
</tbody>
</table>

* The observations recorded in this table were obtained in two separate experiments on cell populations which were concomitantly assessed in radiolabelling studies which employed Fab-adult (Expt. 1) and Fab-SSPE (Expt. 2), respectively.

† All measles virus infections were established in HeLa cells. Lines studied included cells infected acutely with Edmonston strain virus (1) and cells persistently infected with Edmonston strain virus (2), M-VAC attenuated virus (3) or Schwarz attenuated virus (4).

‡ Immunofluorescence (IF) results are expressed as a % of the sampled population expressing surface measles viral antigen.

§ Lysis resulting from incubation of each cell population with measles virus antibody plus complement is expressed as a cytotoxicity index. Background lysis, using heat-inactivated complement, was less than 5 % in each instance.

Our results indicate that total surface viral antigenic expression can be accurately and reproducibly measured by determining saturation plateaus with high titres of radiolabelled, antiviral antibody on viable suspensions of infected cells. Notkins and his associates previously employed radiolabelled antibody to assess relative levels of viral antigenic expression on viable cells infected with vaccinia, influenza and herpes simplex viruses (Hayashi, Rosenthal & Notkins, 1972; Rosenthal, Hayashi & Notkins, 1973). In their studies, radiolabelled antibody was reacted with virus-infected cell monolayers. The use of...
Surface expression of measles virus antigens

Cells in suspension offers theoretical advantages in that (a) cell numbers may be more accurately determined, (b) non-specific trapping of radiolabelled protein is minimized and (c) surfaces of infected cells are more completely exposed.

Antibody binding studies indicated that cells acutely infected with measles virus can express 2 to 3 times more surface viral antigen than persistently infected cells. Also, cells persistently infected with vaccine strains of measles virus tend to express lower levels of surface viral antigen than cells persistently infected with wild type virus. The basis for these differences in surface viral antigenic expression among persistently infected cell lines is not known but may relate to differences in the nature of viral defectiveness among these lines.

Using saturating amounts of specific antiviral antibody we found that $7 \times 10^7$ Fab antibody molecules had to bind per cell in order to effect complete lysis of acutely infected cells. Presumably, the same cells could bind at least $3.5 \times 10^7$ whole IgG molecules. Previous studies in this laboratory (Joseph et al. 1975) indicated that the binding of up to $1 \times 10^6$ measles antibody molecules per cell was insufficient for activating complement-dependent lysis. Those studies likewise indicated that the predominant cytolytic antibody class in patients with SSPE and in persons convalescent from measles virus infection was IgG.

We found that the binding and saturation kinetics for the Fab fragment of measles virus antibody(s) from SSPE patient differed from that observed with the Fab fragment from adult convalescence sera. The reason(s) for the discrepancy is at present unclear. Possible explanations are that responses to measles virus antigens during continuous antigenic stimulation associated with chronic infection (SSPE) differs from the humoral response made following acute infection by (1) restriction of response to various virus antigens (2) affinity of antibody(s) or (3) IgG subclass restriction. These various parameters in immune responsiveness are presently under evaluation.

The observation that persistently infected cells are more refractory to antibody-mediated, complement-dependent lysis than acutely infected cells may be related to the fact that the former express lower levels of measles virus antigen(s) at the cell surface. Our findings in the present investigation have obvious relevance to SSPE in which budding measles virus is poorly expressed at the surface of infected cells even after explantation in culture (ter Meulen et al. 1972). The relative lack of surface viral antigens on SSPE infected cells might allow persistence of such cells despite the presence of a vigorous cytolytic antibody response.

Finally, the ability of vaccine strains of measles virus to cause viral persistence may be of clinical importance since several patients with SSPE have been reported whose only known exposure to measles virus occurred through vaccination (Schneck, Fulginiti & Leestma, 1967; Payne, Baublis & Itabashi, 1969; Parker et al. 1970; Jabbour, Duenas & Modlin, 1975).

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