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(Accepted 30 May 1979)

SUMMARY

Two African green monkey kidney (AGMK) cell lines, 37RC (interferon-producing) and Vero (non-interferon-producing), were infected by egg-grown Sendai virus passaged in eggs at high or low m.o.i. The appearance of haemadsorption, and cytopathic effect (c.p.e.) as well as the presence of haemagglutinating virions in the supernates were much more pronounced with a virus seed obtained with 10⁻³-diluted passages than with a seed obtained with undiluted inoculum. They were also independent of interferon production (data obtained in 37RC and Vero cells were almost superimposable).

In studies carried out with the virus seed prepared at high dilution the establishment of infection was maximal when monolayers were infected as soon as 5 h after trypsinization and cell seeding, regardless of cell density. Virus in supernates obtained from cultures infected 5, 20 or 50 h after seeding exhibited a greatly reduced infectivity for monkey cells, but not apparently for chick embryos. Trypsin treatment of the virus supernates restored their infectivity for AGMK cells more efficiently for virus released from cells infected 5 h after seeding than for virus released from cells infected later after seeding. In keeping with these observations, virus in supernates from cultures infected 5, 20 or 50 h after seeding contained increasing amounts of auto-interfering virions. The decreased infectivity obtained in cell supernates was not accounted for by major differences in virus RNA synthesis. Similarly, the optimum infection established in cells seeded only for 5 h was not due to increased virus adsorption.

Several lines of cells surviving first infection with egg-grown Sendai virus have been obtained and kept in cultures for 3 to 18 months. Virus release and c.p.e. apparently become reduced in the cells surviving the first infection until the newly repopulated monolayers must undergo trypsinization. Weekly protease treatments of the cells reactivate all parameters of virus infection which again will tend to disappear slowly and then reappear following each trypsinization (‘intermittent’ carrier state). The establishment and the ‘intermittent’ reactivation of these lines were not prevented by the inclusion in the medium of anti-Sendai virus serum. Temperature-sensitive ts functions do not seem to play an important role in this virus–host relationship.
INTRODUCTION

Host-induced modifications, mediated by the acquisition of structural materials directly from the plasma membrane of host cells, are rather commonly observed among myxo-(Kates et al. 1961) and paramyxoviruses (Drake & Lay, 1962; Matsumoto & Maeno, 1962; Stenback & Durand, 1963; Klenk & Choppin, 1969; Homma, 1971, 1972; Homma & Ohuchi, 1973; Homma & Tamagawa, 1973; Scheid & Choppin, 1974, 1976; Rima et al. 1977). These changes are often the result of the establishment of persistent infections by these viruses, a phenomenon that also seems easy to induce because these viruses lack the ability to shut-off host macromolecular synthesis. The study of such virus-host relationships is not only relevant to basic virology but also has some direct bearing on the understanding of the pathogenesis of several ‘slow infections’ in humans (Gajdusek, 1977; ter Meulen & Hall, 1978). In the case of multiple sclerosis (MS), for example, if a virus is its causative agent, it remains silent for many years. The host–virus relationships in this as well as in other human diseases, for example the subacute sclerosing panencephalitis (SSPE), must be studied in detail as they may provide clues to the rescue or identification of viruses as candidates for their aetiology.

Sendai virus, or a virus closely related to it, has been proposed as the putative causative agent for MS (ter Meulen et al. 1972; Koprowski & ter Meulen, 1975; Lief et al. 1975). There is also evidence that measles virus may be associated with MS and possibly with other chronic human diseases as well (Pertschuk et al. 1976; Maugh, 1977; Prasad et al. 1977).

Persistent infections by Sendai virus have been reported (Homma, 1971; Kimura et al. 1976; Nishiyama et al. 1976); in murine L cells persistence was seemingly mediated by a host-induced modification of egg-grown Sendai virus that could be abolished by trypsinization of virus suspensions (Homma, 1971). Nishiyama et al. (1976) and Kimura et al. (1976) also described a system where persistent infection was sustained by a ts mutant of Sendai virus unable to cause c.p.e. and endowed with the ability to cause homologous interference with wild-type Sendai virus.

In this paper we report some features of egg-grown Sendai virus infection as well as the establishment of persistent infection in two African green monkey kidney (AGMK) cell lines. Cell-grown Sendai virus exhibited a greatly reduced infectivity for both cell lines, while apparently retaining full infectious potential for embryonated eggs. Trypsin treatment of virions restored the virus infectivity for cells. Both the course of Sendai infection and its persistence in AGMK cells were not dependent upon either interferon (IF) production or the selection of ts mutant viruses. Cell-grown Sendai virus contained auto-interfering particles that were more abundant in supernates obtained from cells infected 50 h after trypsinization and seeding than in those harvested from cells infected 5 h after seeding. In readily established carrier lines, haemadsorption (HAD), release of haemagglutinating (HA) virions in the medium and c.p.e. were modulated by trypsin treatment of the cell monolayers.

METHODS

Cell cultures and media. 37RC and Vero, two AGMK cell lines, were used throughout these studies. 37RC, a line established by Professor G. Rita, then at the University of Siena, Italy, from the kidney of a Cercopithecus aethyops sabeus, has been continuously passaged in our laboratory for many years and produces interferon upon induction with Newcastle disease virus. The Vero cell line (obtained through the courtesy of Dr R. M. Friedman, N.I.H., Bethesda, Md., U.S.A.) does not produce interferon. Both cell lines were grown in Eagle’s minimum essential medium supplemented with non-essential amino acids, 5% foetal calf serum (Eurobio, Paris), antibiotics and 50 μg/ml kanamycin (here referred to as
Cell trypsinization and Sendai virus expression

MEM). Cell maintenance and virus growth were achieved with the same medium, employing a single batch of foetal calf serum devoid of any detectable capacity to inhibit haemagglutination. Mycoplasma contamination was checked by the method of Schneider et al. (1974) after two passages in kanamycin-free medium. Several carrier cultures were obtained by prolonged cultivation of cells surviving Sendai virus infection of 37°C and Vero cells. They were subcultured at 37 °C in MEM, and persistently infected cells have been maintained to date after 70 subcultures over 16 months. In some experiments the cells were grown either at 32 or 39°C.

Viruses. Stocks of Sendai virus (obtained through the courtesy of Dr R. Caliò, Institute of Microbiology, University of Rome) were prepared by allantoic inoculation of 10-day-old embryonated eggs with 0.2 ml of a 10^4 dilution of infected allantoic fluid. After incubation for 3 days at 37 °C, allantoic fluid was harvested, clarified by centrifugation at 4000 g for 10 min and stored at -80 °C.

Conditions of infection. All experiments described involved 37°C and Vero monolayers seeded at 5×10^4 cells/cm^2 and grown either in Linbro plates (16-FB-24-TC) or in Falcon 75 cm^2 flasks. In all cases, trypsinization of monolayers was terminated by addition of equimolar amounts of soybean trypsin inhibitor. Infection was carried out at different times after seeding as indicated in the text. Monolayers were washed with pre-warmed phosphate-buffered saline (PBS) and then virus samples were added in 0.3 ml vol. for the Linbro wells and 1.5 ml vol. for the flasks. After 1 h of incubation at 37 °C (cultures were agitated every 10 min), the virus inoculum was removed, monolayers washed three times with pre-warmed PBS and incubated with 1.5 ml (for Linbro wells) or 10 ml (for the flasks) of MEM. The media were harvested daily and replaced by fresh MEM.

Infectivity titrations. The infectivity for chick embryos was determined by inoculating groups of five embryonated eggs with 10-fold serial dilutions of Sendai virus, and the 50% egg infectious dose (EID_{50}), as measured by spot-checking the haemagglutinating ability of the allantoic fluid, was calculated by the Reed & Muench (1938) method. The infectivity for AGMK cells of Sendai virus was determined by measuring either the haemagglutinating units (HAU) present in the media from, or the haemadsorption by, monolayers infected with 10-fold serial dilutions of virus. Haemagglutination titration was done according to standard procedures. Haemadsorption measurements were made on monolayers grown in Linbro wells (16 mm in diam.) washed three times with PBS supplemented with Ca^{2+} and Mg^{2+} and overlaid with 0.5 ml of 0.1% suspension, in PBS, of human red blood cells (RBC), group O, Rh+. After incubation at 4 °C for 30 min, monolayers were extensively washed with PBS and the extent of erythrocyte adsorption was determined either microscopically or by a benzidine assay. In the former case, with a given optical system (10× ocular, 10× objective), 10 to 15 fields were counted per well and the mean number of haemadsorption foci per field was obtained. By measuring the diameter of a microscopic field in the given optical system, the number of fields per well was calculated to be 1510. Therefore the total number of foci per well is the product of the mean number of foci per field multiplied by 1510. In an attempt to obtain a more precise and rapid estimate of haemadsorption, a benzidine assay was developed. Briefly, monolayers extensively washed with PBS after incubation with RBC, were overlaid with 1 ml/well of 1% benzidine base solution in 90% acetic acid to lyse the erythrocytes adsorbed on to the monolayers. Five min later, samples (from 0.1 ml to the whole 1.0 ml) of the supernatant fluids, normalized to 1.0 ml vol. with the benzidine solution, were added to 1 ml of 3% hydrogen peroxide (36 vol.) and following a colour shift from green to red-brown, 5 ml of 10% acetic acid were added. Absorbance was read at 530 nm in a Leitz Colorimeter and haemoglobin was quantified against a calibration curve with purified haemoglobin. A blank sample consisted of 1 ml of the benzidine solution. Absorbance values of internal controls, consisting of uninfected
monolayers treated as above, were negligible. A linear correlation exists between determinations of haemadsorbing foci and haemoglobin amounts per well over a fivefold range.

Trypsinization of virus suspensions. Virus suspensions were treated with equal volumes of crystalline trypsin solutions in PBS (4 μg/ml, final concentration; Worthington Biochemicals) for 5 min at 37 °C. Replicate virus samples were diluted with equal volumes of PBS. Equimolar concentrations of soybean trypsin inhibitor were then added to both mixtures which were centrifuged at 20000 g for 40 min. The pellets were redissolved in MEM and tested for haemagglutinating activity and infectivity.

Virus labelling and purification. Virus labelling was achieved as follows: (i) 10 day-old embryonated eggs were given 200 μCi of ³H-uridine at the time of infection; (ii) 37RC cultures, infected with the 10⁻³-diluted egg-seed (as in Fig. 1) were given 10 μCi/ml at the end of the adsorption period. Both allantoic fluids and supernates were clarified at 5000 rev/min for 10 min and spun at 40000 g in a Spinco ultracentrifuge for 45 min. Virus pellets, resuspended in Veronal buffer were purified according to Kato (1967). Crude stock preparations were concentrated by ultracentrifugation and layered on top of a discontinuous 5% to 60% sucrose gradient in Veronal buffer and centrifuged at 20000 rev/min for 20 min in a Spinco SW-39 rotor. Fractions (0.4 ml) were collected from the bottom of the tube and assayed for haemagglutination activity. Fractions of the peak were pooled.

Virus adsorption assay. Radioactive egg- and cell-grown virus suspensions in MEM were added to 37RC monolayers seeded in Linbro plates 5 and 50 h earlier (0.2 ml/well). At different time intervals, monolayers were washed three times with PBS and lysed with SDS buffer (NaCl, 0.1 M; MgCl₂, 0.015 M; tris-HCl, 0.01 M; SDS, 0.5%; EDTA, 0.5 M, pH 7.0). Samples were dissolved in Bray's solution and counted in a liquid scintillation spectrometer.

Interferon titrations. Infected and uninfected culture fluids were checked for interferon presence by the ability to reduce by 50% plaque formation by vesicular stomatitis virus on CV-1 cells (an AGMK line). Any Sendai virus present was removed by adjusting the media to pH 2 (5 days at 4 °C).

RESULTS

Influence of the passage history of the virus seed on the growth of egg-grown Sendai virus in 37RC and Vero cells

37RC and Vero monolayers grown in 75 cm² Falcon flasks were infected 12 to 18 h after cell seeding at an input multiplicity of 30 HAU/10⁶ cells, with several virus stocks prepared as follows: the standard virus stock (see Methods) was passaged in embryonated eggs either undiluted for three passages (undiluted seed) or at a 10⁻¹ dilution twice (10⁻¹-diluted seed) or at 10⁻³ dilution twice (10⁻³-diluted seed). Control cultures received the same amount of allantoic fluid from uninfected eggs. Daily after infection, culture fluids were harvested, assayed for haemagglutinating activity as well as for the presence of interferon and replaced by fresh medium. Replicate cultures were checked for haemadsorption by microscopic evaluation of foci and then trypsinized in order to determine the number of cells still adhering to the surface.

Fig. 1 shows the data obtained in a representative experiment. In 37RC cell cultures virus release, c.p.e. and cell death, as measured by counting viable (trypan blue dye-excluding) cells, as well as haemadsorption assessment, all showed a coherent pattern that can be summarized as follows: (i) with the 10⁻³-diluted virus seed, all parameters apparently peaked at 48 h p.i. except cell death; haemadsorption determinations showed a diffuse pattern 48 h p.i. which declined thereafter and fully disappeared on day 10; (ii) with the other two virus seeds, the changes of all parameters were less prominent than with the 10⁻³-diluted seed. In Vero cells, virus release, c.p.e. and cell death were less pronounced than
Cell trypsinization and Sendai virus expression

Fig. 1. Time-course study of (a) 37RC and (b) Vero cultures infected with various egg-grown Sendai virus seeds. ○, 10⁻³-diluted seed; ●, 10⁻¹-diluted seed; △, undiluted seed. For seed identification, see text. ———, HAU released into the medium/10⁶ cells. ——, Percentage of cells detached from the plastic surface. C.p.e. was graded from 0 (zero) to 4 depending on the percentage of cells exhibiting c.p.e. The maximal degeneration (4) signifies that over 75% of cells exhibited c.p.e.

in 37RC cultures, whereas haemadsorption assessments were more diffuse in Vero than in 37RC cells. Upon microscopic examinations, the c.p.e. observed in both cell lines was characterized by cell rounding and subsequent detachment of a variable fraction of cells. Those surviving the acute phase of infection showed an arachnoid-like framework that could last as long as 5 days. Eventually these cells would fully recover from virus infection and slowly repopulate the cultures. No syncytia were observed. Daily titrations of IF revealed the presence of low amounts of IF in media from 37RC cultures harvested on days 1 and 2 (Fig. 6). Both the kinetics of IF production in 37RC cultures and its absence in media from Vero cultures (as already shown by Desmyter et al. (1968) indicate that IF does not play an important role in the modulation of this virus–host relationship.

Since the c.p.e. observed in cells infected either with the 10⁻¹ or with the undiluted virus seeds did not apparently attain the 100% mark, the following experiments studying the features of infection of 37RC and Vero cells with egg-grown Sendai virus were carried out using the 10⁻³-diluted seed.

Reduced infectivity of cell-grown Sendai virus for cell cultures and its restoration by treatment with trypsin

Fluids harvested daily from 37RC and Vero cultures infected by Sendai virus (as shown in Fig. 1) were tested for infectivity for embryonated eggs, 37RC and Vero cells. All fluids showed greatly reduced infectivity for both cell lines, while exhibiting unaltered infectious
potentials for eggs. Supernates harvested at 48 h from 37RC and Vero cultures infected with 10^-8-diluted seed were therefore studied in some detail. Cell-grown Sendai virus samples showed a 4 log reduction of infectivity when tested on cells, as compared with embryonated eggs. This host-induced change of infectivity was abolished by trypsin treatment of virus suspensions, in keeping with previous observations (Homma, 1971; Scheid & Choppin, 1974). It must be stressed that under the conditions used trypsin treatment of virions did not cause any significant loss of HAU.

**Influence of the length of time between cell trypsinization and virus infection on the growth of egg-grown Sendai virus in 37RC and Vero cells**

During experiments leading to the establishment and characterization of persistently infected cultures of 37RC and Vero cells, it was noted that treatment of monolayers with trypsin for subculturing was an important factor in modulating virus release into the media (see later). It was of interest, therefore, to investigate whether the age (i.e. the time interval from trypsinization) of monolayers at the time of infection would play any role in the replication of Sendai virus. Accordingly, 37RC and Vero cultures were trypsinized and cells seeded at a multiplicity of 5 x 10^4/cm². At 5, 20 and 50 h later, the cultures were infected at an m.o.i. of 30 HAU/10⁶ cells with the 10^-8-diluted egg-grown virus seed. Cultures were then studied as already described for the experiments in Fig. 1. Cells infected only 5 h after seeding released haemagglutinating virions into the media at a rate slightly higher than that of cells infected 20 h and much higher than that of cells infected 50 h after seeding. The general pattern of infection closely paralleled that described in Fig. 2. In addition, at 48 h p.i., haemadsorption occurred in diffuse areas for the 5 h and 20 h samples, but only as discrete foci in the 50 h samples; c.p.e. assessments (see Legend to Fig. 1) were close to 1 in the 50 h sample and to 3 and 4 for the 20 h and 5 h samples, respectively. The fact that also in this experiment the data obtained in 37RC and Vero cultures were superimposable, provides additional evidence that IF is not an important factor in this virus-host relationship.

**Characterization of virus present in media harvested from cells infected at various time intervals after trypsinization**

Media harvested 48 h p.i. of 37RC cultures in the experiments described in the preceding section were studied in some detail. They were: 37RC 5 h, 37RC 20 h and 37RC 50 h supernates.

All supernatants, given at the m.o.i. of 50 HAU/10⁶ cells, were found to be poorly infectious when titrated by haemadsorption on the homologous cell line; trypsin treatment of the samples (see Methods) prior to titration, however, restored their infectivity more efficiently for the 5 h (3.5 log₁₀ gain) than for the 20 h (1.7 log₁₀ gain) and the 50 h (1.4 log₁₀ gain) supernates. It appears that the infectivity of a fraction of virions from the 20 h and the 50 h supernates could not be restored by trypsin treatment, suggesting that the 20 h and 50 h supernates may contain increasing amounts of auto-interfering non-infectious virions resistant to protease treatment.

This was shown to be true by the interference experiment described in Fig. 2. The correct amount of egg-grown Sendai virus to be used for the interference experiment was determined (Fig. 2a). The 10^-8 dilution was chosen as the minimum amount of virus capable of inducing 100% diffuse haemadsorption 24 h p.i. The 37RC 5 h, 20 h and 50 h supernates were ultracentrifuged at 20000 g for 40 min. Pellets resuspended in MEM were adjusted at 100 HAU/ml and then diluted 1:2, 10^-1, 10^-2 and 10^-3. Then 0.5 ml of each diluted sample was mixed with an equal volume of 10^-3 dilution of egg-grown Sendai virus. The mixtures were incubated on 37RC monolayers in Linbro wells, seeded 20 h earlier. After 24 h, haemadsorption was assessed by the benzidine assay (see Methods) and the data are given
Cell trypsinization and Sendai virus expression

Fig. 2(a) Titration curve of egg-grown Sendai virus on 37RC cells as measured by the benzidine method for haemadsorption determinations. (b) Interference test between egg-grown and cell-grown Sendai viruses assayed on 37RC cell cultures (for details, see Methods). Reduction of haemadsorption by interfering viruses is given as the percent ratio of haemadsorption (as determined by the benzidine method) induced by mixtures of egg- and cell-grown Sendai viruses as compared to haemadsorption induced by egg-grown virus only:

\[
\frac{\text{egg-grown Sendai + 37RC-grown Sendai infection}}{\text{egg-grown Sendai + MEM infection}} \times 100.
\]

The cells were infected with virus 5 h (○—○), 20 h (□—□) and 50 h (△—△) after trypsinization at the final dilutions shown.

In Fig. 2(b) as the percent ratio of haemadsorption assessed in cultures infected with the various mixtures compared to haemadsorption observed in cultures infected with the egg-grown Sendai virus diluted with an equal volume of MEM.

In all instances mixing egg-grown and cell-grown Sendai viruses caused a reduction of total haemadsorption observed. This reduction was dependent upon the dilutions of cell-grown viruses and was more pronounced whenever high concentrations of 20 h and 50 h supernates were used. These data indicate that all cell-grown virus preparations contain auto-interfering sedimentable particles, but these seem to be particularly abundant in the 20 h and 50 h sample.

Virus RNA synthesis in 37RC cells infected with egg-grown Sendai virus

In view of the preceding observations indicating that significantly less virus was released by cells infected 50 h as compared to 5 h after seeding, it was of interest to study virus RNA synthesis in these cells. 37RC monolayers, seeded 5 and 50 h earlier, were infected with egg-grown Sendai virus (30 HAU/10^6 cells), in the presence of 2 μg/ml of actinomycin D and 10 μCi/ml of ^3H-uridine added at the end of the adsorption period. One day later, the medium was removed, monolayers repeatedly washed and then scraped off the plastic using a rubber policeman. Equal numbers of cells were lysed in SDS buffer (NaCl, 0.1 M; MgCl₂, 0.0015 M; tris-HCl, 0.01 M; SDS, 0.5% and EDTA, 0.5 M, pH 7.0). RNA was extracted by the chloroform–isoamyl alcohol–phenol technique (Penman, 1966), ethanol precipitated,
dissolved in the above SDS buffer, except for SDS made 0.1%, layered on top of a linear 15 to 30% sucrose gradient, prepared in the same buffer and centrifuged for 16 h at 23000 rev/min in a Spinco SW-25.1 rotor. Fractions were collected with an ISCO system. All fractions were TCA precipitated and the radioactivity counted in a Beckman liquid scintillation apparatus. Fig. 3 shows the profiles of RNAs extracted from the cells that had been infected 5 h (Fig. 3a) and 50 h (Fig. 3b) after seeding. No appreciable differences (both qualitatively and quantitatively) can be seen, which indicates that virus RNA synthesis proceeds unaltered in both conditions. RNA from uninfected cells treated in the same way showed a radioactivity profile close to background.

Adsorption capacity of egg-grown and cell-grown Sendai viruses to 37RC cells seeded 5 and 50 h earlier

Equal amounts of 3H-uridine-labelled and purified egg- and 37RC-grown Sendai viruses were added to cell monolayers 5 h and 50 h after cell trypsinization and seeding. All kinetic curves reached a plateau at 50 min and were essentially identical.

Establishment of persistently infected 37RC and Vero lines showing an 'intermittent' expression of virus infection

In Fig. 1 it is apparent that virus infection tends to slow down after the 48 h peak. Under all conditions tested, cells surviving first infection with egg-grown Sendai virus slowly regained normal morphology and growth potential so that cultures were repopulated. Ten to 15 days p.i. no HAU could be detected in the media, and c.p.e. was absent. At every weekly passage, 37RC and Vero cultures grown in Falcon flasks were subjected to two types of protocol. The first one consisted of exposing the monolayers to trypsin (Difco; 1:250, 0.1%) plus EDTA (0.04%). Cell detachment was attained in about 3 min, trypsin action was stopped by the addition of equimolar concentrations of soybean trypsin inhibitor, the cells were washed and reseeded in fresh MEM at the routine density in Falcon flasks to ensure the continuous passage of the persistently infected cell line. The HAU released into the media, the c.p.e. and the growth patterns of the monolayers were assessed daily. The second protocol consisted of treating replicate cultures with 10⁻² M-EDTA for about 10 min at 37°C until the cells were detached from the surface. After washing out EDTA,
equal numbers of cells either received no further treatment or were treated with trypsin (1:250, 0.1%)
for 3 min at room temperature. Both samples were then given the soybean trypsin inhibitor, washed and
reseeded in Linbro plates. These cell populations, the EDTA- and the EDTA÷trypsin-treated cells, were used to assess haemadsorption by the benzidine assay 48 h after seeding. The data are shown in Fig. 4. At each trypsinization of 37RC
cultures, substantial amounts of HAU were detected in the media with a peak at 48 h (Fig. 4a). In the EDTA÷trypsin-treated 37RC cells seeded in Linbro plates, an intense and
diffuse haemadsorption was observed 48 h after trypsinization. In 37RC cells treated with
EDTA only, haemadsorption was much less pronounced (Fig. 4b). HAU values released
into the media declined after the 48 h peak. At the morphological examination, c.p.e. was
evident 48 h after trypsinization, but then slowly declined. Haemadsorption assessments
carried out occasionally on day 5 showed either a fully negative picture or only rare isolated
foci. The pattern exhibited by persistently infected Vero cultures (Fig. 4c) after each
weekly trypsinization closely paralleled that described for 37RC cultures except that the
amounts of HAU released into the media were either very low or absent.

The virus released into the media of 37RC 'intermittent' carrier cultures exhibited at each
subpassage an almost complete loss of infectivity for 37RC and Vero cells, while retaining full infectious potential for embryonated eggs. Trypsin treatment of the samples restored their infectivity for the cells (data not shown).

Altogether, seven 37RC and four Vero carrier cultures have been established and kept in continuous growth for 3 to 18 months. The patterns of virus expression of these cultures were always superimposable on to those shown in Fig. 4. It is noteworthy that after at least 1 month of subcultivation the amounts of HAU released into the supernates at the 48 h peak tended to decrease.

Trypsin-mediated reactivation of virus expression was documented in all cultures except for a 37RC carrier line that was apparently fully ‘cured’ after 5 weeks (no HAU release and negative haemadsorption).

Persistently infected 37RC cultures have been tested for their ability to sustain re-infection by the parental egg-grown Sendai virus. Briefly, densely confluent ‘carrier’ and untreated 37RC monolayers were infected with $10^{-3}$-diluted egg-grown Sendai virus at a m.o.i. of 100 HAU/10⁶ cells. The release of HAU into the media as well as cell damage was assessed daily. Yields of HAU from ‘carrier’ cultures were low, within the range usually observed and much lower than the yields obtained from control 37RC cultures infected with egg-grown Sendai virus. Accordingly, control cultures exhibited extensive cell destruction, whereas c.p.e. was practically absent in the superinfected ‘carrier’ cultures (Fig. 5).

**Attempts to cure the persistently infected 37RC cultures**

The inclusion of rabbit anti-Sendai virus serum in the culture medium was examined for its effect on the persistent infection of 37RC carrier cultures. A 0.5 ml amount of undiluted serum, capable of inhibiting 512 HAU of egg Sendai virus and with a neutralizing titre of 1:1024 was added to 5 ml of MEM at each cell passage in a 25 cm² Falcon flask. After 1 month of culture in anti-Sendai virus serum, the cells were grown for one passage in serum-free medium and then assayed for haemadsorption foci in Linbro plates. The data demonstrate that the number of foci detected in all instances was not modified by the previous treatment with the antiserum. It seems reasonable to imply that the role of extracellular virus is not an important one in maintaining the carrier state.

The effects of temperature shifts of cell cultures on virus expression were also studied.

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*Fig. 5.* Time-course study of superinfection of 37RC ‘carrier’ cultures with egg-grown Sendai virus. ○, Control 37RC cultures; □, ‘carrier’ 37RC cultures. ---, HAU released daily into the supernates; -- —, percentage of cells detached from the plastic surface.
Table I. Number of haemadsorbing foci (15 to 20 cells) per microscopic field in co-cultivation cultures of (EDTA- or EDTA + trypsin-treated) 37RC carrier cells and of normal 37RC cells detached either with EDTA or with EDTA + trypsin

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* EDTA at \(10^{-2}\) M for 10 min at 37 °C.
† EDTA (0.04 %)+trypsin (Difco; 1:250, 0.1 %) for 3 min at room temperature.
§ A 0.5 ml amount of anti-Sendai virus serum was added to 0.5 ml of MEM at the time of seeding. Haemadsorption foci were counted 4 h after removal of the antiserum by extensive washings in MEM, addition of fresh MEM and incubation at 37 °C. Control cultures (i.e. devoid of antiserum) were subjected to the same washings, addition of fresh medium and incubation for 4 h.

Carrier cultures of 37RC cells were shifted from 37 °C, the usual incubation temperature, either to 32 °C or to 39 °C and grown at these temperatures for two passages. No significant differences were observed between the 'shifted' cultures and the replicates kept at the usual temperature, indicating that the patterns of virus release, haemadsorption and c.p.e. were not apparently controlled by a \(ts\) mutation either in the virus or in the cell genome.

Trypsin-mediated reactivation of virus expression is not accounted for by re-infection

The observation of a progressive decline of HAU release into the media following a number of subpassages, as well as the apparent 'cure' of one carrier line, suggested that protracted subculturing was selecting either for 'cured' cells or for cells still infected but no longer 'inducible' to virus expression by trypsin treatment. In both instances, the reactivation of virus production observed after each trypsinization could be interpreted as being due to re-infection of 'cured' or trypsin-insensitive cells by trypsin-activated virions budding from those cells that were still virus producers. One would then expect that 'carrier' and normal cells, both treated with EDTA + trypsin and then co-cultivated, would exhibit a higher number of haemadsorption foci in the absence of anti-Sendai virus serum than would replicate cultures grown in the presence of serum. The experiment testing this hypothesis is described in Table I. Cells derived from either carrier or normal 37RC cultures were scraped off the plastic surface using a rubber policeman and treated with either \(10^{-2}\) M-EDTA or EDTA (0.04 %)+trypsin (0.1 %) for 3 min at room temperature. Soybean trypsin inhibitor was added to all cell samples which were then washed and reseeded in every possible combination in Linbro plates. Total cell inoculum was \(1 \times 10^6\) cells/ml and equal amounts of cells were mixed. Haemadsorption foci were scored 48 h after cell seeding (for details, see Table I). The data indicate that the amounts of foci were critically related to the type of treatment received by the 'infected' cells (EDTA + trypsin being much more effective than EDTA alone). The treatment received by the normal cells did not play a significant role. Furthermore, the inclusion of anti-Sendai virus serum to the culture medium at the time of cell seeding was irrelevant.

It may be concluded, therefore, that virus reactivation resulting from trypsinization of 'infected' monolayers is not apparently mediated by re-infection of 'cured' cells by means of trypsin-reactivated virions.
Fig. 6. Kinetics of IF production in 37RC and Vero cultures infected by egg-grown Sendai virus. Conditions of infection are those described in Fig. 1 for 10^{-3}-diluted seed. (a) HAU released into the medium by 37RC (○—○) and Vero (●—●) cultures. (b) IF units/ml detected in the supernates of the same 37RC (○) and Vero (●) cultures.

Lack of influence of IF during the course of Sendai virus infection and the establishment of 'intermittent' carrier cultures

Figure 6 shows the kinetics of IF production in two representative experiments carried out with 37RC and Vero cells during either the course of first infection or the establishment of an 'intermittent' carrier culture by Sendai virus. Supernates from Vero cultures were free of IF throughout, as previously shown by Desmyter et al. (1968), confirming the non-inducibility of this cell line to IF production. As far as 37RC cultures were concerned, it is noteworthy that substantial amounts of IF were detected only early p.i. and declined thereafter. The kinetics of IF production and of HAU release do not apparently suggest a significant role of IF in the decline of HAU released into the media. In addition, no detectable IF was found whenever a burst of haemagglutinating virions was detected in the media following each trypsinization and subculturing of the 37RC intermittent carrier cell lines. This experiment has been repeated four times with 37RC and twice with Vero cultures. These data, therefore, demonstrate that the course of Sendai virus infection of 37RC and Vero cell cultures as well as that of the 'intermittent' carrier cultures derived thereof, is not apparently controlled by IF production.

Absence of ts mutants of Sendai virus in 'intermittent' carrier cultures

The emergence of ts mutants of Sendai virus during the establishment of persistently infected cultures has been reported (Kimura et al. 1976; Nishiyama et al. 1976; Collins & Flanagan, 1978). Although the results obtained from shifting cultures either up or down (reported earlier) did not show any influence of the incubation temperature on virus production by 37RC carrier cultures, the ability of virus released by such cultures to infect normal 37RC cells at different temperatures was tested. Virus suspensions obtained from a carrier culture that had been propagated for over 1 year were titrated by haemadsorption in
37RC monolayers at 32, 37 and 39 °C. Virus samples were either untreated or subjected to trypsin treatment prior to infection. Haemadsorbing foci were scored 48 h p.i. The titres from cultures given untreated virus samples were negligible throughout, as was to be expected in view of the very poor infectivity of cell-grown Sendai virus. Titres from cultures infected with trypsin-treated virus samples and kept at different temperatures did not vary by more than 1 log. It appears, therefore, that Sendai virus populations produced by 37RC carrier cultures do not contain a significant amount of ts mutant virions.

DISCUSSION

The data described in the present paper provide evidence for a trypsin-mediated modulation of virus expression in Sendai virus-infected AGMK cells. The action of trypsin on the growth of Sendai virus in murine L cells was reported by Homma (1971, 1972). Briefly, it was observed that a direct action of trypsin on L cell-grown Sendai virus restored its infectivity for L cells (Homma, 1971), as well as its haemolytic (Homma, 1972) and fusion activities (Homma & Tamagawa, 1973). The biological defectiveness of L cell- and HeLa cell-grown Sendai virus was found to be correlated with the presence of reduced amounts of one virion glycopolypeptide (GP4) and of increased amounts of another one (GP2), as compared with fully infectious egg-grown Sendai. A mild trypsin treatment of cell-grown virions caused, at the same time, a conversion of cell-borne virus to a biologically active form with a concomitant increase of GP4 (Homma & Ohuchi, 1973). Similar data from Scheid & Choppin (1974) were obtained with Sendai virus grown in MDBK cells. Cell-borne virions were found to lack glycoprotein F0 which is a precursor of glycoprotein F1. Cleavage of the former into the latter, which occurs spontaneously in ovo, was achieved in vitro by treatment of virions with trypsin, thus restoring the haemolysing and cell-fusing activities as well as the infectivity of Sendai virus for cells in culture. Classes of mutagenized Sendai virus mutants also exhibiting activation by other proteases, have been described (Scheid & Choppin, 1976). We have also observed that trypsin treatment of cell-grown virions restores their infectivity for the AGMK cells tested.

Under our experimental conditions, if infection is carried out shortly after cell trypsinization, there appears to be an influence on virus expression, i.e. virus release, formation of haemadsorbing foci and c.p.e. were increased (compare cells infected 5 h and 50 h after seeding). Trypsinization of persistently infected cultures at a stage at which virus release and haemadsorption were undetectable also resulted in a substantial activation of both processes (Fig. 4). The re-infection of 'cured' cells is ruled out by the experiments carried out in the presence of a vast excess of anti-Sendai virus serum. The markedly reduced virus expression in cells infected 50 h after cell trypsinization is also not due to an altered virus RNA synthesis both in terms of RNA species and virus RNA accumulation in the cytoplasm (Fig. 3). Adsorption, too, does not apparently play a major role, since both egg- and cell-grown virions adsorbed equally well to 'young' (5 h after seeding) and 'old' cells. In combination, these data point to virus maturation and/or export as the most likely step to be influenced by trypsin treatment.

The evidence indicating that IF is not an important factor in the virus-host relationships described here is rather convincing (Fig. 6). Persistently infected cultures differing with respect to IF production and response to exogenous IF have been described (Collins & Flanagan, 1977).

Auto-interfering 'defective' virions have been reported in several virus-host systems (Kingsbury et al. 1970; Norrby et al. 1970; Rima et al. 1977) and have been thought to play a role in the establishment of persistently infected cultures (Huang & Baltimore, 1970). They have often been obtained by passaging undiluted virus inocula. We have also documented the presence of such interfering Sendai virus particles (Fig. 2) which may also have
played a role in the outcome of the experiment described in Fig. 1 (undiluted versus \(10^{-3}\)-
diluted seed). The data shown in Fig. 2 indicate that substantially higher amounts of sedimentable interfering particles were present in the 37°C 50 h than in the 5 h supernatants. This is apparently in keeping with all other data pointing to some kind of trypsin-related effects as factors modulating virus expression. Studies aimed at elucidating the biochemistry and structure of these particles are in progress.

The mechanisms of action of trypsin under these experimental conditions are at present unknown. The activation of an endocellular protease by trypsin treatment of cells has been reported (Werb & Aggeler, 1978). This cell protease could provide a specific host-dependent cleavage of virus glycoproteins responsible for infectivity. However, this does not seem to be the case in the system described here, since trypsinization of persistently infected carrier cultures activated virus production, yet does not induce the release of fully infectious virions, as one would expect.

In conclusion, the increasing ‘age’ of the cultures (as measured by the length of the time interval between cell seeding and infection) seems to affect the life cycle of Sendai virus in AGMK cells (i) by reducing virus yields into the media and (ii) by causing the production of auto-interfering particles that are even resistant to a subsequent trypsin treatment in vitro. These changes may be brought about by trypsin-mediated change either of the glycoprotein components of the plasma membrane or of structures such as microfilaments and microtubules that are involved in the process of cell rounding, detachment, reseeding and flattening. A striking decrease of actin-containing microfilaments has been observed in cultures of epithelial cells and fibroblasts infected with Sendai virus and other paramyxoviruses (Fagraeus et al. 1978). The same mechanisms may play an important role in the ‘intermittent’ virus expression of persistently infected cultures. It cannot be excluded that phenomena of this kind may be operative in the virus reactivations occurring in humans during the course of prolonged persistent infections.

This work was supported in part by grants from Consiglio Nazionale delle Ricerche, Progetto Finalizzato Virus (NN. 78.00349.84, 78.00389.84 and 78.00393.84) and from N.A.T.O. (No. 1152). The authors are indebted to Mrs A. Tamburrini for skilful secretarial assistance.

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Cell trypsinization and Sendai virus expression


(Received 8 January 1979)