5-BROMODEOXYURIDINE-INDUCED FORMATION OF VIRUS-LIKE PARTICLES IN NAEGLERIA GRUBERI EG$_S$

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SUMMARY

Exposure of axenic cultures of the amoeboflagellate Naegleria gruberi EG$_S$ to the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) resulted in the induction of virus-like particles (VLP) and various structures associated with their development and presumed transmission. Previously, VLP induction could be accomplished only by growing amoebae in the presence of living bacteria as a food source. Addition of excess thymidine along with BrdU did not block induction of particles. This account demonstrates that the EG$_S$-VLP system responded to BrdU as do a number of mammalian cell lines harbouring latent viruses, and provides the basis for future work on the infectivity of the VLPs for other amoebae as well as tissue culture cells.

INTRODUCTION

The EG$_S$ strain of the amoeboflagellate Naegleria gruberi has been shown to harbour virus-like particles (Schuster, 1969). A definite sequence of stages was reported in the development, release, and possible transmission of these particles in the amoeba culture (Schuster & Dunnebacke, 1971, 1976). The factors responsible for inducing formation of virus-like particles (VLP) were of particular interest in that amoebae grown axenically showed no evidence of particles; transfer of such cells to a medium with living bacteria as a food source resulted in appearance of particles, initially in the nucleus and subsequently in the cytoplasm. Over a period of ca. 6 days, the time varying with culture conditions (fluid or agar cultures, growth temperature, abundance of bacterial food supply, size of amoeba inoculum, etc.) virtually all of the cells in the culture underwent lysis. Examination of washings stained with phosphotungstic acid from agar plates containing lysed cultures revealed particles with a virus-like appearance (Schuster & Dunnebacke, 1977). About 10% of cells on the plate survived as cysts, but examination of these in the transmission electron microscope revealed no VLPs. An initial assumption that bacteria were the source of the VLPs was apparently ruled out by the ability of any of several different edible strains (Aerobacter aerogenes, Escherichia coli) to induce particle formation when used as a food source (Schuster, 1969).

This paper reports on the ability to induce VLPs and all subsequent developmental stages in axenic cultures of the EG$_S$ strain through the use of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU). This account: (1) establishes the endogenous nature

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of the VLPs in the EG₅ strain of *N. gruberi*; (2) demonstrates that a protistan system is responsive to the same techniques successfully employed in virus induction in a variety of mammalian cell lines; and (3) provides the basis for an approach to studying the infectivity of VLPs for other amoebae and tissue culture cells.

**MATERIAL AND METHODS**

**Cultivation of amoebae**

*N. gruberi* EG₅ stocks were maintained axenically in a yeast extract-peptone-liver extract medium containing 10% v/v heat-inactivated foetal calf serum*. Bacterized cultures, established periodically from axenic stocks to check on the presence of VLPs, were grown on an agar-containing yeast extract-peptone-glucose medium with *Aerobacter aerogenes* as the bacterial food source. All cultures were maintained at 21 °C. Other *N. gruberi* strains employed in this study included a sister strain of EG₅, EG₅₆, found not to have VLPs when grown in the presence of bacteria (Schuster & Dunnebacke, 1974), the S strain, and CR 54. In addition, pathogenic *N. fowleri* strains (Carter, HB-3, and O360) were studied, though these strains were grown at 37 °C.

**BrdU treatment**

Axenic cultures were grown in 125-ml, screw-capped Erlenmeyer or Fernbach flasks containing a shallow layer of medium (10 or 20 ml, respectively), using log phase cells as an inoculum. In experiments where concentration effects of BrdU on cell populations were examined, inocula were adjusted to give an initial concentration of ca. 10³ amoebae/ml. Counting of cells was done with a Coulter counter (model ZB). BrdU (Sigma Chemical) was added after 48 h of growth, to give a final concentration in the culture medium of 100 μg/ml. BrdU stock solutions were prepared fresh as needed and filter-sterilized before addition to cultures by passage through a 0.45-μm Millipore membrane; all BrdU used in these experiments came from a single batch. Non-BrdU-containing control cultures were run with all experiments. In one experiment, thymidine (Sigma Chemical) was added to the amoeba population along with BrdU at 10-fold the BrdU concentration.

**Microscopy**

Amoebae were fixed for electron microscopy 7–10 days following addition of BrdU. Cells were harvested from growth flasks, fixed in collidine-buffered 2% v/v glutaraldehyde, postfixed in veronal-acetate-buffered OsO₄, stained with aqueous uranyl acetate, dehydrated through graded ethanols, and embedded in Maraglas. Sections were stained with lead citrate and examined in a Philips 300 or Zeiss 9S-2 electron microscope operating at 80 or 60 kV, respectively. Living cells were examined and photographed using a Zeiss microscope equipped with differential interference optics.

**RESULTS**

**Growth experiments**

Growth of EG₅ in BrdU at 100 μg/ml gave populations with approximately half the number of amoebae/ml found in control populations (Fig. 1). Amoebae of the EG₅ strain appeared more sensitive to BrdU, with a roughly 5-fold difference observed.

- Axenic cultures of the EG₅ strain are now available from the American Type Culture Collection (ATCC No. 30540). In co-operation with Dr Pierre-Marc Daggett of the ATCC, we have checked their stock of EG₅ for particles after cryopreservation and found development of VLPs in response to bacterial induction.
in cells/ml when BrdU and control populations were compared (data not plotted). Thus, in spite of the reported toxicity of BrdU for many cell types particularly where prolonged exposure was part of the experimental design, the relatively high concentration employed in this study was inhibitory but not lethal for EG amoebae.

**Structural effects**

At the ultrastructural level, control cells showed all aspects of typical morphology: nucleus with central nucleolus, mitochondria, vesicles of endoplasmic reticulum, contractile vacuole, etc. (Fig. 2). With perhaps one exception (see below), no unusual structures or organellar modifications were observed and at no time was any evidence seen of VLPs or their developmental stages as previously described (Schuster & Dunnebacke, 1971).

Light-microscopic examination of amoebae exposed to BrdU for up to 2 weeks showed no pronounced abnormalities (Figs. 3, 4) other than an unusually large number of vacuoles seen in some of the cells. Some nuclei had an inflated appearance suggestive of an early stage in VLP formation (Fig. 4). The general shape of amoebae as well as the manner of movement seemed normal. Examination of BrdU-treated amoebae in the electron microscope showed a number of distinct morphological modifications exclusive of those related to VLPs. Since these were seen in most of the strains examined, they are described as primary effects of BrdU on the cells. Nuclei appeared enlarged and irregular in shape, mitochondria were frequently vacuolated (Fig. 10) or showed bizarre modifications (Fig. 11) and were often seen in varying degrees of degeneration; vesiculation of endoplasmic reticulum was more pronounced than in control cells, and food vacuoles containing ingested amoebae were observed. Of all these
changes noted, the mitochondrial alterations were the most reliable indicator of BrdU effect.

VLP induction

Secondary effects were those associated with VLPs and related structures. Nuclei contained densely granular clusters often, but not always, in the region of the nucleolus (Figs. 5, 7). Particles enclosed by walls, similar to those previously described as VLPs, were seen free in the perinucleolar area or in association with presumed generative sites (Fig. 5). The nuclear envelope was found to possess tubes which were apparent exit channels for VLPs from the nuclear compartment. Particles were also found free in the cytoplasm and associated with vacuolated mitochondria (Fig. 10) or with membranes apparently forming about cytoplasmic granular regions. Fibrils, not seen in cells from control populations, were found in both nuclear and cytoplasmic compartments (Figs. 5, 8). Finally, spheres surrounded by a microtubule-like fringe and containing VLPs were found outside of the amoebae (Fig. 9). In short, all aspects of amoebic VLPs induced by bacterial feeding (Fig. 6) were observed in axenic cultures containing BrdU.

Comparison of VLPs and associated structures produced by BrdU treatment (Fig. 5) with those induced by feeding upon bacteria (Fig. 6) revealed some differences. Bacteria-induced particles were generally uniform, consisting of a wall enclosing a dense core; these particles were associated with nucleoplasmic generative bodies during early developmental stages. BrdU-induced particles tended toward irregularity; wall structures lacking the dense core were not infrequently seen, in addition to masses of apparent core material lacking bounding walls (Fig. 7). In bacteria-fed populations, the nucleolus appeared free of involvement in VLP formation, most of this activity taking place in the perinucleolar region. In BrdU-treated populations, the nucleolus often consisted either wholly or in part of dense spheres (Fig. 7). It was not clear whether these spheres ultimately became encapsulated to form VLPs. Also, there was a higher incidence of cytoplasmic fibrils in BrdU-exposed cells than those from bacteria-fed populations (Figs. 5, 8).

The EG U strain of *N. gruberi*, a sister strain of EG S apparently free of VLPs when grown in the presence of bacteria (Schuster & Dunnebacke, 1974), when exposed to BrdU revealed none of the overt signs of VLP presence (fibrils, particles, spheres, etc.). This strain did exhibit, however, spherical masses within the nuclei of cells similar to those found in the EG S amoebae (cf. Figs. 7 and 12). These spheres may have been
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representative of an early stage in VLP formation, or may simply have formed as a nonspecific response to BrdU. *N. fowleri* strains exposed to BrdU for up to 5 days at 37 °C showed no evidence of spherical masses in the nucleus or any other aspects of VLP formation. The same was true of the other *N. gruberi* strains (S and CR 54) employed in this study.

It was previously noted that control cells appeared normal with one exception. A structure that showed up with regularity in both BrdU-treated and control populations of the EGs and EGb strains, possibly related to VLPs, is seen in Fig. 13. This structure consisted typically of a densely granular region associated with spheres about the size of VLPs, the whole complex lying within the cytoplasm in a membrane-bound vesicle. Their appearance, though suggestive of involvement in VLP formation, may be a manifestation of aging of the amoeba population. No such structures were seen in the S strain of *N. gruberi*, though CR 54 amoebae exposed to BrdU showed comparable formations; they were not observed in BrdU-treated *N. fowleri* strains. The presence of these dense complexes in BrdU-treated populations of EGs amoebae appeared to vary inversely with VLP developmental stages; in those populations where all aspects of VLP development were observed (stages up to and including VLP-containing extracellular spheres), these bodies were seen either infrequently or not at all.

In the single experiment in which thymidine was added in excess (1000 μg/ml) along with the BrdU (100 μg/ml) to a culture of EGs amoebae, development of VLPs and related structures was not blocked. In fact, the incidence of cells exhibiting VLPs was higher than in the non-thymidine-containing BrdU control culture.

Cultures varied in stages of VLP development over the time period at which they were fixed. All BrdU-treated cultures of EGs showed VLPs having an intranuclear location, usually in about 10% of the cells examined in sections. In some preparations, about 30% of the cells showed evidence of some aspect of VLP development. Both the BrdU-treated cultures used for determining growth response to BrdU concentration as plotted in Fig. 1 were fixed and examined in the electron microscope. The cultures, though essentially identical in terms of cell number, time of exposure to BrdU, growth temperature, etc., showed variation in VLP development. One of the cultures had cells with intranuclear VLPs only; cells of the other culture contained intranuclear, cytoplasmic, and extracellular stages in VLP formation.

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Figs. 5, 6. VLP induction by BrdU (Fig. 5) and bacteria (Fig. 6) compared.

Fig. 5. Amoebae from axenic culture, exposed to BrdU for 7 days. Nucleus (n) appears swollen and contained VLPs (v), nucleolar material of varying density, a generative body for VLPs (g) and fibrils (fm). Exit tubes for VLPs can be seen on the nuclear envelope (arrow). Fibrils can also be seen in the cytoplasm (fc), as can membranous structures (mb) forming about cytoplasmic VLPs. Note vacuolation of the mitochondria. x 15,000.

Fig. 6. EGs amoeba grown in the presence of living bacteria for induction of VLPs. The nucleus is inflated and contains VLPs (v). Exit tubes are seen on nuclear envelope (arrows). A membranous body (mb) is apparently forming in cytoplasm. x 15,000.
DISCUSSION

BrdU has been used for induction of virus particles in a number of mammalian tissue culture systems, where a characteristic pattern of low incidence existed in the occurrence of particles (Aaronson, Todaro & Scolnick, 1971; Dahlberg, Perk & Dalton, 1974; Gerber, 1972; Grimley, Barry & Schaff, 1973; Hsiung, 1972; Lowy, Rowe, Teich & Hartley, 1971; Margalith et al. 1975; Rhim et al. 1973; Rowe, Lowy, Teich & Hartley, 1972). The present study dealt with a protistan system in which VLPs and related structures formed as part of a well defined, reproducible sequence of events. The trigger for VLP development was transfer of axenically grown amoebae to a medium where living bacteria served as a food source. The mechanism by which induction occurred was unknown, though it was speculated that it might have been due to a possible difference in DNA content of non-VLP-containing axenic vs. VLP-containing bacteria-fed amoebae (Schuster & Dunnebacke, 1976).

BrdU-induced appearance of VLPs in axenic cultures of EG₈ amoebae is unquestionable. In some dozen experiments in which BrdU was added to axenic cultures, one or more of the stages in VLP development were observed. Less clear were the results obtained using BrdU on axenic cultures of EG₈, a non-VLP-containing sister strain of EG₈ (Schuster & Dunnebacke, 1974). BrdU-exposed EG₈ amoebae formed nuclear particles lacking walls but none of the other stages of VLP development as observed in EG₈ was ever seen. Other strains of *Naegleria* spp. examined showed neither nuclear masses (as did EG₈) nor any other VLP-associated structures. Appearance of vacuolar dense masses in the cytoplasm occurred in both of the EG strains examined, whether or not cells were exposed to BrdU. In the absence of any positive indication that these structures were involved with VLP development, we conclude that these masses were representative of some aspect of cell aging in our populations.

Variability in numbers of amoebae exhibiting VLPs was typical of previous findings working with bacteria-fed cultures (Schuster & Dunnebacke, 1976). In such systems, the greatest incidence of cells with VLPs was found when amoebae were grown on an agar surface covered with a uniform growth of bacteria. When amoebae were grown in the same bacterized medium minus agar (i.e., a fluid medium with suspended bacteria), VLP induction was delayed and erratic. The present study made use of axenic fluid cultures and, while it is not possible to equate axenic and bacterized culture systems,
the same factors that reduced VLP incidence in amoebae in fluid bacterized media might also have been operating in fluid axenic media. In part, these factors would include relative nutrient levels, O₂ availability, density of amoebae, and so on. For technical reasons, we have not tried growing EG₅ amoebae axenically on BrdU-containing agar surfaces. We have, however, transferred BrdU-exposed axenic EG₅ cells to saline agar overlaid with semi-solid phage assay agar in an effort to observe plaque formation (unpublished observations). Plaques were not observed due to the mobility of the amoebae, but amoebae lysis was evident when the BrdU-treated culture was compared with the control.

Some subtle differences were found between VLP cycles as induced by BrdU and by bacteria. In BrdU cultures, many VLPs in the nucleus lacked cores and there was considerable variability in size of the intranuclear spheres (Fig. 7) which may or may not have been destined to become VLPs. There also appeared to be a greater incidence of cytoplasmic fibrils than seen in amoebae grown in the presence of bacteria.

The concentration of halogenated pyrimidine used in this study (100 μg/ml) was unphysiologically high when compared to BrdU levels employed for virus induction in mammalian tissue culture systems. The same was true for duration of exposure which, in our study, was 7–10 days. For mammalian cell studies, concentrations ranged from 4 μg/ml for 4 days (Margalith et al. 1975) to 300 μg/ml for 1 day (Aaronson et al. 1971). Margalith et al. found that BrdU concentrations of 50 μg/ml and higher were toxic for BALB/3T3 cells. A maximum response of 8% in lymphoid cell lines was reported by Gerber (1972) to a BrdU concentration of 25 μg/ml over 7 days. Unlike tissue cultures where uptake of materials from the growth medium by cells apparently poses no particular problem, Naegleria amoebae do not readily incorporate specific compounds from growth media owing perhaps to permeability barriers or to the presence of precursor pools of materials that delay or minimize drug effects (Fulton, 1970; Preston & O’Dell, 1971). The appearance of VLPs in the EG₅ strain was one indication of BrdU entrance into amoebae, and ultrastructural alterations seen

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Fig. 10. Mitochondrion from EG₅ amoeba exposed to BrdU. Note swollen appearance, and presence of dense material in mitochondrial vacuole or pocket. Such configurations were frequently observed in cells from BrdU cultures. × 61,000.

Fig. 11. Mitochondrion from BrdU-exposed amoeba, showing unusual zipper-like formation in matrix. Such alterations of mitochondrial morphology were never observed in control amoebae. × 61,000.

Fig. 12. Portion of nucleus of EG₅ amoeba exposed to BrdU for 7 days. Note presence of dense spheres (cf. with EG₅ nucleus in Fig. 7). These may represent VLPs induced by BrdU, though other aspects of VLP development were never observed in EG₅ amoebae. × 43,000.

Fig. 13. Dense material seen in cytoplasmic vacuole of EG₅ amoeba exposed to BrdU. Spheres (arrows) suggestive of VLPs are seen, but these structures are thought to represent some aspect of cell aging since they were found in both BrdU-treated and control populations × 58,000.
in amoeba mitochondria was another. Iododeoxyuridine has also been reported capable of virus induction in the same cell lines where BrdU was effective (Aaronson et al. 1971; Lowy et al. 1971), but no VLPs were observed after limited efforts in the use of this substance on axenic EG₈ amoebae (unpublished observations). Fluorodeoxyuridine, which was also examined as a means of VLP induction, was highly toxic to the amoebae.

Virus production was successfully blocked in mammalian cell systems by addition of thymidine (Gerber, 1972; Grimley et al. 1973; Lowy et al. 1971), suggesting that the mechanism of action was at the level of BrdU incorporation into DNA. Inability to block VLP development by addition of excess thymidine in the present study may rule out this particular explanation as a viable mode of action. Considering, however, the previously noted permeability barriers encountered in Naegleria and the difficulty in demonstrating [³H]thymidine uptake into axenic amoeba cultures after exposure for 4 days (unpublished observations), this explanation may still have some validity.

Another point remaining unresolved is whether all EG₈ amoebae harbour latent VLPs or whether VLP spread through a culture occurs when VLPs are activated in a small number of amoebae, with subsequent spread of particles taking place from cell-to-cell. While ultrastructural evidence supports a horizontal (i.e., cell-to-cell) spread of VLPs (Schuster & Dunnebacke, 1976), the results obtained with BrdU do not exclude the possibility that every EG₈ amoeba is capable of producing its own VLPs given the appropriate activator and sufficient time for expression. Furthermore, our results from the use of BrdU on axenic EG₈ amoebae populations suggest that this strain might also contain unexpressed VLPs. This is of interest in that it seemingly rules out a previous suggestion that the VLPs were acquired while the EG₈ strain was cultivated in the presence of chicken embryo extract (Schuster, 1969), to which the EG₈ strain had never been exposed. BrdU-induced 'particles' (Fig. 12) in the EG₈ strain are different from VLPs and associated structures found in EG₈.

The ability to induce VLPs in axenic cultures of EG₈ amoebae provides distinct advantages in the study of this protozoon-VLP system not yet available in other protistan systems (Diamond & Mattern, 1976). Previously, any attempt to analyse the EG₈-VLP system was complicated by the presence of bacteria in the preparations. Triggering VLP development with BrdU makes it possible to test the infectivity of these particles for axenic EG₈ amoebae not exposed to BrdU, for other Naegleria strains and species, for other genera of soil amoebae (Acanthamoeba, Dictyostelium etc.) and, finally for mammalian cells lines.

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