MORPHOLOGICAL RESPONSE OF CULTURED CELLS TO
NAEGLERIA AMOEBA CYTOPATHOGENIC MATERIAL

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SUMMARY

Naegleria amoebae contain cytopathogenic material (NACM). The morphological response of cultured cells to this material follows a number of characteristics in common with those resulting from infectious agents. The cytopathologic changes varied depending on the strain of the cultured cells. Among those from 17 different vertebrate sources, both primary and continuous cell lines, some were destroyed completely by dilutions of NACM up to $10^{-8}$ while others appeared unaffected by NACM at any concentration. The response had no apparent relationship to species, organ source, or passage level of the cells. The reaction was typified by a long latent period (4—10 days) during which the number of cells in the culture increased up to 10-fold, followed abruptly by a short period (less than 24 h) during which all of the cells were destroyed. The latent period was prolonged when the culture conditions were adverse, or when the amount of NACM in the inoculum was minimal. A high multiplicity of NACM in the inoculum lysed the entire culture, while dilutions near the end-point caused generalized or only focal changes of rounded cytopathic cells. The cytopathic effect could be maintained in cultured cells by serial passage, such that the total activity greatly exceeded what could be attributed to the original inoculum. These findings are consistent with the concept that NACM has properties of an infectious agent and that its quantity is enhanced and spread through the culture by cell-to-cell contact and by cell division.

INTRODUCTION

Naegleria amoebae contain a cytopathogenic material (NACM, nā'-cm; Naegleria amoeba cytopathogenic material) that has been characterized as an infectious agent (Dunnebacke & Schuster, 1971; Schuster & Dunnebacke, 1974). A variety of amoeba genera have been examined for their ability to produce cytopathology in tissue culture monolayers. Thus far, only Naegleria amoebae — including strains pathogenic for humans — have been found to contain the cytopathogenic material that destroyed avian and mammalian cells (Dunnebacke & Schuster, 1974; Schuster & Dunnebacke, 1976). In previous studies, we have reported on the apparent replication of NACM based on the increase in total NACM activity over the original inoculum as demonstrated by cumulative dilutions through a series of passage steps in cultures undergoing lysis (Dunnebacke & Schuster, 1977a).

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Key words: Naegleria, cytopathogenic material.
Many questions, however, have remained concerning the properties of NACM and its ability to produce cytopathology. The NACM unit is of small size and lacks a demonstrable nucleic acid component (Dunnebacke & Schuster, 1977a,b; 1978; Dunnebacke, 1982). Further characterization of NACM will be the basis of a subsequent manuscript (T. H. Dunnebacke & J. S. Dixon, unpublished). In this paper, we address the question of the nature of the NACM-induced cytopathic effect and present new observations that shed light on this phenomenon: (1) a comparison of crude NACM-containing amoeba extracts with purified NACM prepared by column chromatography; (2) a characterization of specific cellular pathology in tissue culture monolayers based on the NACM dilutions used; and (3) a demonstration of cell-to-cell spread of NACM-induced cytopathology in tissue culture monolayers. These observations provide supporting evidence that NACM behaves like an infectious agent.

MATERIALS AND METHODS

Cell cultures

A variety of cell cultures were used to determine the range of cell types that responded morphologically to NACM. They included preparations from 10-day-old chick embryos and 16-day-old mouse embryos, cultures of rat glioma (C6, CCL 107), iguana heart (IgH-2), human embryonic brain (Flow Laboratories), human astrocytoma (H4), human neuroblastoma (IMR-32), mink lung and HeLa cells. Additional cultures donated by members of this laboratory (YRDL) included human foetal kidney (K845), human foetal lung (L645), African green monkey kidney (Vero), human skin (Y2, passage 7), baby hamster kidney (BSK, passage 121), mouse fibroblasts (3T3, passage 56), rabbit kidney (RK-13, passage 24), and normal rat kidney (NRK-L, passage 11). An account of the use of some of these cultures was included in a conference report (Schuster & Dunnebacke, 1980).

Cells were cultivated in Eagle's minimal essential medium with 10% foetal bovine serum with some exceptions: (1) chick cells were grown in medium 199 with 1% chick serum and 1% foetal bovine serum; (2) rat glioma and human embryonic brain cells were cultured in HEPES-buffered medium 199 containing either 5% or 10% foetal bovine serum. Iguana heart cells were maintained at room temperature; the others were incubated at 37°C in a humidified 5% CO2 atmosphere. Antibiotics, streptomycin and penicillin (200 units/ml), were routinely added to media. Serum was heat-inactivated before use.

Cells from trypsinized stock cultures were counted in a Coulter Counter (model FN) and seeded at a concentration of 100,000 cells per ml medium. After incubation for 24 h, samples of NACM in a series of dilutions in tissue culture medium were added directly to the cultures and incubation was continued. Uninoculated cultures served as controls. Cultures grown with 10% foetal bovine serum were inoculated directly, or the medium was replaced with that containing 2% serum before the addition of NACM. All cultures were observed daily with an inverted light microscope, and morphological changes noted. Selected cultures were photographed live by phase contrast, or after fixation by air drying and staining with Stat Stain (VWR Scientific, Inc) with the aid of an inverted microscope (Zeiss ICM 405).

Fluids and cell debris from selected cultures were collected and stored frozen at −20°C until their use in additional assays, or for the serial passage of NACM in cultured cells.

Amoeba cultures

Amoebae of the EGs strain of Naegleria gruberi obtained from a California soil sample (Schuster, 1963) have served as a standard for the NACM preparations. NACM prepared from N. jadini, 0400,
Cells and Naegleria amoeba cytopathogenic material

and N. fowleri MB-41 (HB-1) from a human in Florida, NF-66 from a human in Australia, PA-90 from a water source in Australia, 0359 and 0360 from humans in Belgium and 6088 from a human in California, who survived primary amoebic meningoencephalitis, were used for comparative purposes.

Amoebae were cultivated axenically in 32-ounce prescription bottles in 30 ml of medium consisting of yeast extract/peptone/liver concentrates plus 10% foetal bovine serum (Balamuth, 1964). At intervals of 4–7 days, the medium was decanted, the cells adhering to the surface were scraped free with a rubber 'policeman' and collected after centrifuging, for storage as frozen pellets. Fresh medium was added to the cells remaining in the bottles for further incubation until the next harvest.

Amoebal lysates

The collected amoebae were subjected to repeated freeze-thawing (4 times), and the resulting lysate was clarified by centrifugation at 10000 g for 30 min. The supernatant fluid was passed through a 0.45 μm Millipore filter and concentrated 10-fold by lyophilization (Dunnebacke & Schuster, 1977a). This material, designated as crude amoebal lysate, was divided into samples and stored at -20°C.

NACM purification

Biologically active NACM, as determined by assay on chick embryo cells, was subjected to a series of fractionations by column chromatography including gel filtration on agarose Bio Gel A-0.5m, ion exchange on DEAE Bio Gel A and CM Bio Gel A, separation on hydroxylapatite Bio Gel HTP, (Bio Rad Laboratories), and chromatofocusing on PBE 94 (Pharmacia Laboratories). These procedures effectively separated biologically active NACM in good yield from other amoebic components. Relative purity was assessed by the ratio of NACM titre to absorbance at 280 nm. Fractions used in this study and designated as purified had relative purities compared to the crude material in excess of 1:50 000. Details of the isolation procedure will be presented in a separate paper.

RESULTS

NACM cytopathology

The responsiveness of the cultures was the same to NACM whether prepared from the non-pathogenic or from the pathogenic species of the Naegleria amoebae (Table 1), and to NACM from crude amoebal extracts or from purified fractions.

The cytopathogenic response to NACM was a highly specific reaction that occurred in a selection of cell cultures derived from a variety of sources (Table 1). Two features of the response were related to the specific cell cultures: (1) the sensitivity as measured by the titre of a standard NACM sample and (2) the length of the latent period. Rat glioma and chick embryo cells had the shortest latent periods and demonstrated the highest titres of NACM. Some cell types, such as human embryo brain and baby hamster kidney, had longer latent periods and consistently lower titres than those obtained in chick or rat glioma cells. In other cells tested, only minimal or questionable responses were noted: those that required more than 10 days to develop and those that occurred only at the highest concentrations of NACM. Cells from some sources appeared to be completely unaffected. The addition of serum in medium at 2% or 10% concentration after the addition of NACM caused no difference in the response of the cells.
Cultures of rat glioma cells increased sevenfold in cell number in both control and treated cultures before the first changes were noted. On the fourth day, gaps resulting from the retreat of cell processes and cell-rounding appeared exclusively in the monolayers inoculated with NACM (Fig. 1A,B). On the following days, such gaps were accentuated by the appearance of many dense cells and large holes in the cell sheet (Fig. 1C,D). By the sixth day, the cell sheet was completely destroyed (Fig. 1E,F). Replacement of the culture medium failed to revive any cells that might have been among the cytopathic remnants adhering to the surface of the culture dish. In contrast, during this period, the control cultures maintained viable monolayers.

Human embryonic brain cells seeded into culture dishes increased threefold before the onset of NACM cytopathology 6 days after inoculation. By the seventh day, total breakdown of the inoculated cultures was evident (Fig. 1G,H). The baby hamster kidney cells multiplied 10-fold before they were destroyed 7–8 days after inoculation.

Chick embryo cell cultures show a rapid growth and, unless seeded sparsely, can overgrow and slough off the surface before the NACM cytopathic response develops. Under conditions used here, with sparse seeding, the cells form a confluent sheet.

### Table 1. Response of cell cultures to NACM

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Naegleria gruberi</th>
<th>jadini</th>
<th>EGS</th>
<th>400</th>
<th>MB-41</th>
<th>NF-66</th>
<th>PA-90</th>
<th>0359</th>
<th>0360</th>
<th>6088</th>
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<td>8†</td>
<td>7</td>
<td>8</td>
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<td>8</td>
<td>8</td>
<td>7</td>
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<td>8</td>
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<tr>
<td>Rat glioma</td>
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<td>7–7</td>
<td>8</td>
<td>7</td>
<td>6–7</td>
<td>7–7</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Human embryo brain</td>
<td>6</td>
<td>4–7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>2</td>
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<td>5–7</td>
<td>4–7</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>–</td>
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<td>Baby hamster kidney</td>
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<td>4–7</td>
<td>2</td>
<td>5</td>
<td>5–7</td>
<td>4–7</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
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<td>4–7</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Human astrocytoma</td>
<td>10†</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Human foetal kidney</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
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</tr>
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<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Green monkey kidney</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>Rabbit kidney</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Human foetal lung</td>
<td>&gt;14</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Human neuroblastoma</td>
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<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Human skin</td>
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<td>2</td>
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<tr>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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</table>

Cultures from the sources listed seeded at 100 000 cells per ml medium, incubated for 24 h, were inoculated with NACM from a standard preparation of the amoeba strain at dilutions ranging from 10^{-2} to 10^{-8}. The first observable difference between the inoculated and the uninoculated control cultures occurred between 4 and 10 days for the susceptible cultures; this was designated the latent period*. The titre of the NACM preparation determined by the last dilution in a series to cause complete destruction of the culture, an end-point equivalent to an LD_{100}, is given as the inverse logarithm of the dilution†. Cultures showing no response to NACM are indicated by 0; cultures not inoculated are indicated by –.
within 3–4 days that, with no medium change, can be maintained intact for some 21 days. Although the number of scattered rounded degenerative cells increases with age, the massive cell killing related to NACM is readily apparent. The time interval before the response is related to the amount of NACM in the inoculum; massive doses resulted in the shortest latent periods of 4–6 days; exposure to small quantities of NACM resulted in latent periods of 10–13 days (Table 2). Culture conditions such as incubation temperatures below 37 °C or incubation in medium that was acidic lengthened the latent period. Conditions that prolonged the latent period had little or no effect on the sensitivity of the cultures; that is, on the ultimate titre of a standard NACM sample. Chick embryo cells in medium at pH 6-8 required 10 days to yield a response comparable to one that occurred at 5 days for cultures of the same cell preparation in medium at pH 7.5.

Why some cell cultures are destroyed by NACM and others are not is unknown. No pattern for the cytopathic response was discerned that related to the cell species or organ source of the cells, whether normal or tumorous, slow or fast growing, fibroblastic or epithelial, or related to the passage level of the culture.

**NACM assay**

Chick embryo cells were used for NACM assays. Starting at a 10⁻² dilution in tissue culture medium, NACM samples at 10-fold dilution steps from crude or purified preparations were added to the media of chick cell cultures. No subsequent media

<table>
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<th>Days of incubation</th>
<th>Dilution of NACM sample</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
<th>10⁻⁷</th>
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<tr>
<td>4</td>
<td>N*</td>
<td>N</td>
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<td>N</td>
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<tr>
<td>5</td>
<td>Lysed†</td>
<td>Lysed</td>
<td>Lysed</td>
<td>Lysed</td>
<td>Lysed</td>
<td>N</td>
<td>N</td>
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<tr>
<td>6</td>
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<td>Lysed</td>
<td>Lysed</td>
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<td>Rounded§</td>
<td>N</td>
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<tr>
<td>7–14</td>
<td>Lysed</td>
<td>Lysed</td>
<td>Lysed</td>
<td>Lysed</td>
<td>Lysed</td>
<td>Rounded</td>
<td>N</td>
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<table>
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<th>Dilution of NACM sample (×10⁻³)</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
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<td>4</td>
<td>N</td>
<td>N</td>
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<td>5</td>
<td>Lysed</td>
<td>Lysed</td>
<td>?§</td>
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<td>6</td>
<td>Lysed</td>
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<td>Rounded</td>
<td>?2 Foci</td>
<td></td>
<td>N</td>
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<td>7–14</td>
<td>Lysed</td>
<td>Lysed</td>
<td>Rounded</td>
<td>2 Foci</td>
<td></td>
<td>1 Focus</td>
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</tbody>
</table>

The appearance of cultures following inoculation with serial dilutions of a highly purified sample of NACM on the indicated days of incubation.

* N, sheet of normal-looking cells.
† Lysed cytopathic cells.
§ Questionable appearance.
|| Foci of rounded cytopathic cells.
changes were made since this contributed to culture overgrowth and sloughing of the cell sheet during the NACM latent period. No effect related to amoeba cytotoxins was noted at these concentrations (Chang, 1974; Cursons, Brown & Keys, 1978; Visvesvara & Callaway, 1974). In selected samples near the critical endpoint of the NACM activity, or in serial passage in cell cultures, dilutions of the inoculum were made at twofold steps (Table 2).

At the time of inoculation, one day after seeding, the cells in the cultures were sparse and well separated (Fig. 2A,B). During the next few days the cells increased in numbers and formed a confluent cell sheet (Fig. 2C,D). After confluency, the mitotic activity was reduced and cultures unaffected by NACM remained more or less static in appearance for long intervals. Some degenerating cells were present in most cultures and increased in numbers with age (Fig. 2E,G). At first the inoculated cultures were indistinguishable morphologically from the uninoculated controls (Fig. 2C,D). Abruptly after 4 days, and within 24 h, the NACM response became evident (Fig. 2F,H). Within a series of 10-fold dilution steps, a sharp break-point existed between those cultures that did, and those cultures that did not, show complete cellular destruction. This breakpoint, equivalent to a lethal dose, $LD_{100}$, was used as the first measure of the titre of a sample whose cytopathic effect might extend through dilutions of $10^{-2}$ to $10^{-6}$ (Tables 1, 2).

A few hours before destruction, subtle changes could be noted in the inoculated cells. The refractility of the sheet as seen with the light microscope increased, and spaces became apparent between individual cells. Along the edge of some cells, blebs of cytoplasmatic material could be seen. At this stage, the endoplasmic reticulum of cells observed in the electron microscope appeared swollen and engorged with fine granular material (Dunnebacke & Schuster, 1974).

The appearance of the destroyed cultures was related to the relative amounts of NACM in the inoculum. Invariably, cultures destroyed at, or near, the end-point in a dilution series consisted of dense, rounded, highly refractile cells that remained attached to the surface of the culture dish (Table 2; Fig. 2H). In cultures destroyed by large multiplicities of NACM, the residual debris consisted of elongated remnants of the lysed fibroblasts with a scattering of rounded cells (Table 2; Fig. 2F). Examination of unfixed or stained cultures showed that the nuclear material was condensed into a dark mass in the lysed cells. In the rounded cells, figures that appeared to be arrested

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Fig. 1. Cells growing on the surface of Petri plates were photographed with phase-contrast illumination on the stage of an inverted microscope. A-C.E. An uninoculated culture of rat glioma cells showing the normal growth pattern and maintenance of cell sheet, photographed on day 4, 5 and 6, respectively. B,D,F. A companion culture of rat glioma cells inoculated with NACM and photographed on day 4, 5 and 6, respectively. On day 4, the initial cytopathic change can be seen by the retraction of the processes of some cells resulting in the formation of a hole in the sheet of cells. By the 5th day, the disruptions in the cell sheet are larger, and by the 6th day, all of the cells in the culture have been destroyed. The remnants on the culture surface are not viable cells. G. A control culture of human embryonic brain cells displaying the normal growth pattern on day 7. H. A companion culture of human embryonic brain cells inoculated with NACM, at day 7, shows extensive cytopathic damage. X250.
Fig. 2
Cells and Naegleria amoeba cytopathogenic material

abnormal mitotic structures were frequent. Attempts to revitalize the cytopathic cells by a change of medium have invariably failed. The response to vital stains, Trypan Blue and Neutral Red, indicated that these cells were dead.

Near the end-point of the NACM activity, particularly in samples diluted in small increments, distinct regions of rounded cells could be seen on otherwise normal-looking cell sheets (Fig. 3). Over the next 2–3 days, such regions expanded in area and in numbers of involved cells producing a plaque of cytopathic cells. In the presence of vital stains, the cells in the centres of the foci appeared to be dead. The normal-appearing cells surviving outside and between the foci, were alive and could be stimulated to grow when the medium was changed.

Serial passage of NACM

The supernatant fluids at 5 to 10-fold dilution steps from cytopathic cultures of rat glioma, human embryonic brain, baby hamster kidney, and chick embryo cells were filtered and placed onto new cultures. Cytopathic effects were produced that were similar to the initial NACM cytopathology. In rat glioma cells, the serial transfer of the NACM cytopathogen was carried through five steps with complete cell destruction at each passage. In each successive passage, however, approximately 1 day was added to the time of onset of pathology. By the fifth passage in both the control and the experimental cultures, the increased evidence of senescence after 9 or 10 days made it difficult to distinguish specific from non-specific culture changes. In cultures of human embryonic brain and in baby hamster kidney cells, the prominence of non-specific degenerating cells plus the long latent periods made the passage of NACM difficult to distinguish beyond three or four steps. Cytopathic materials were interchanged in passages between rat glioma and human embryonic brain cells, and between baby hamster kidney and chick embryo cells. All subsequent changes were typical of NACM cytopathology. The long maintenance period of lightly seeded cultures of chick embryo cells made them best suited for serial passaging of NACM.

Comparable dilutions of NACM from crude amoebal lysate and from purified NACM (Table 3) were inoculated into sparsely seeded chick embryo cultures. Passages of fluids and cell debris from frozen-thawed uninoculated cultures served as controls. All materials selected for passages were stored frozen, and thawed just before

Fig. 2. Cultures of chick embryo cells from a NACM assay, air-dried, stained with Stat Stain, and photographed on an inverted microscope (Zeiss, ICM 405); ×200. Left column, uninoculated control cultures; right column, inoculated cultures; A,B, cultures at the time of inoculation showing the sparsity of cells; C,D, cultures 4 days after the time of inoculation showing the increase in cell numbers and the formation of a confluent cell sheet; E,F, by 5 days, the intact control cultures are starkly contrasted to the lysed remnants of the cytopathic cells (l) exposed to many units of NACM; G,H, by 6 days, a small increase in the number of non-specific degenerating cells in the control cultures can be seen and contrasted with the rounded cytopathic cells (r) and destruction of the inoculated cultures at, or near the end-point in a dilution series with 10-fold steps of a NACM sample (see Table 2). Little additional change is noted with extended incubation other than the increase in non-specific degenerating cells that coincide with senescence in the control and in the non-cytopathic cultures.
use. Unlike the lysates prepared directly from amoebae, the frozen–thawed materials from cultured cells contained no cytotoxins and it was possible to observe responses that corresponded to the pattern of NACM cytopathology following inoculations at low dilutions even after extended latent periods.

The state of purification of the NACM used in the original inoculum had no effect on the response observed during serial passage. In each case, the pattern of cytopathology followed that at, or near, the dilution end-point in the assays already described. The rounded cytopathic cells were generally first noted as foci in the cell sheet (Fig. 3). Although response to NACM at each passage step was evident only at dilutions prepared in small increments, the accumulated amount of NACM as measured over a number of steps greatly exceeded the quantity of NACM in the original inoculum. The rate of inactivation of purified NACM in culture medium at 37 °C, identical to that from crude preparations, was about 50% per day. At this rate, less than 1% of the NACM in the original inoculum would be active after 7 days at 37 °C. In the serial passages described in detail in Table 3 the NACM activity was maintained through seven and nine passage steps for 76 and 106 days, respectively, at 37 °C. It seems very unlikely that any residual NACM that may have been present in the original inoculum could have persisted and been detected after such an extended time at 37 °C. On this basis, the NACM activity found in the passaged cultures must have resulted from the production of additional active material in the affected cells.

In an attempt to enhance the NACM response, after four serial steps the next three passages (Table 3) were made using unfiltered material. In these, small clumps of cell debris could be seen after transfer on the surface of the new cell sheet. The cell debris did not inhibit or affect the growth of the new cells surrounding it (Fig. 4A). However, at the end of 8–10 days, in the immediate vicinity of the debris, refractile, shrinking and rounded cells became apparent (Fig. 4B). On the next day, a distinct ring of lysed cytopathic cells surrounding the debris was encircled by a band of rounded cytopathic cells that extended out into the normal-appearing sheet of fibroblasts (Fig. 4C). The area of the cytopathic cells continued to increase for 2–3 days. Individual fibroblasts at the edge of the expanding ring could be seen within a few hours to change into shrunken, rounded, cell remnants when observed with the light microscope.

In cultures containing cytopathic cells adjacent to the debris, the debris was removed by rocking the medium in the dish, or with a pipette. The cytopathic cells adhering to the dish were scraped free and stored frozen. When placed onto new cultures, again, localized cytopathic responses occurred in the regions of the transferred cell debris (Table 3).

Fig. 3. Foci of rounded cytopathic cells on sheets of cells that otherwise appear to be unaffected. A. Early appearance of focus; B, focus at 6th passage step indicated in Table 3; C, focus 3 days after first observation; D, focus in same passage series as in B, fixed and stained 7 days after it was first observed. It increased in size for 3 days, then appeared to remain static. A, B, D. Fixed and stained; a number of the cells in the lesions were washed off during the staining process; C, phase-contrast micrograph. ×300.
Table 3. Passage of NACM in chick embryo cells

<table>
<thead>
<tr>
<th>Passage (sample no.)</th>
<th>Crude lysate*</th>
<th>Purified sample†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dilution</td>
<td>Days to CP</td>
</tr>
<tr>
<td>1 (101)</td>
<td>10000</td>
<td>6</td>
</tr>
<tr>
<td>2 (113)</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>3 (146)</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>4 (195)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>5 (254)†</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>6 (310)†</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>7 (357)†</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>8 (403)</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>9 (503)</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Summation of dilutions: 6·2×10⁹

Summation of days at 37°C: 1×10⁹

Serial passages of NACM in chick embryo cultures.

* NACM from crude extract of amoebae with a starting titre (LD₅₀₀₀) of 10 000.
† NACM following gel filtration, ion exchange, and chromatofocusing, purification factor greater than ×50 000, with a starting titre of 10 000.
‡ Passaged fluid was unfiltered and contained clumps of debris from the frozen–thawed cytopathic cells.
§ Photograph of culture in Fig. 4B,c.
||Photograph of culture in Fig. 3B,D; type of cytopathic cells: CP, cytopathology; R, rounded cytopathic cells; FR, foci of rounded cytopathic cells; Sp, spot or lesion of cytopathic cells adjacent to passaged tissue debris.

The enlargement of the cytopathic focus, or the region adjacent to tissue debris was the same in cultures with liquid medium or in cultures that had been overlayed with hard agar following inoculation.

DISCUSSION

The cytopathology caused by NACM is separate from that related to the presence of viable amoebae, microorganisms, mycoplasmas or cytotoxins. It is not associated with virus-like particles. The small size and protein nature of NACM distinguish it from...
Fig. 4
known viruses (Dunnebacke & Schuster, 1971, 1974, 1977a). Along with these differences, new observations of the morphological response of cultured cells to NACM reported here show that it shares many features with infectious agents. These include: (1) specificity of the cytopathic effect in cell cultures such that some cell lines respond and some do not; (2) correlation between the amount of the agent in the inoculum with the time and the cytological appearance of the affected cells; (3) the formation and expansion of discrete areas, or foci, of affected cells; (4) cell-to-cell spread of the effect in a cell sheet; and (5) most important, the enhancement of the active response through serial passages in cultures, such that the total amount of the measurable activity obtained far exceeds that in the original inoculum.

A salient feature of all NACM–cell interactions is the long latent period during which the numbers of cells in the cultures multiply. Thus, cells that respond cytopathically and die are not the same cells present at inoculation but, rather, they are the progeny cells. In consideration that: (1) the response of the lysed cytopathic and the rounded cytopathic cells are related to the amount of NACM in the inoculum; (2) the amount of NACM in the medium is progressively reduced by heat inactivation (Dunnebacke & Schuster, 1977a); and (3) the synchrony in the time of appearance of the cytopathic cells, we propose that the initial NACM–cell interaction takes place in an early period after inoculation in those parental cells present at that time, and, as the numbers of cells increase, new NACM material is produced and distributed to the daughter cells.

Cytopathic foci occur when the amount of NACM in the inoculum is insufficient to become associated with each cell present at that time. It is likely that, as with viral infections, each focus represents the result of a single NACM–cell interaction and the final localized response observed occurs as the result of the further distribution of NACM in each succeeding generation of daughter cells.

The fact that NACM is present in the cytopathic cells is shown by the serial passages from cytopathic cultures and by the localized response in regions adjacent to debris from passaged cytopathic cultures. A considerable amount of NACM material had been retained in the cell remnants following lysis and freeze–thawing. Possibly, more effective separation procedures will show a close relationship between the amounts of NACM and the stage of cellular change following inoculation.

The localization of new cytopathic cells in the region of cell debris occurred in spite of the fact that the cultures were in liquid medium and subjected to turbulence as they were removed from the incubator for microscopic examination on a daily basis. The presence of the lysed cytopathic cells adjacent to the debris and the rounded cytopathic cells at the periphery of the lesions further indicates that the NACM factor had a cell-to-cell distribution during the latent period.

During passage in any cell system, there is the possibility that a latent virus, or agent, within the cell may be stimulated and that the resulting effects are from the stimulated agent instead of the inoculating agent. This possibility seems unlikely as an explanation of NACM activity, because passages have been accomplished with NACM prepared from a variety of Naegleria strains, and passages have been accomplished in rat glioma, human embryonic brain, baby hamster kidney, and in a
Cells and Naegleria amoeba cytopathogenic material

number of cultures prepared from chick embryos. In addition, passages begun in one cell line have been continued in other cell lines.

On the basis of morphological observations, the pattern of the activity of NACM in cultured cells is compatible with that of an infectious agent in that it can stimulate a process in cells that in turn results in the production of more cytopathogenic material. The similarities of the response of cells to NACM from crude amoebal extract and to NACM subjected to the purification procedures shows that the biologically active ingredient in the purified product is not the result of some extraneous component in the amoebae or in the cell culture materials. NACM is a biologically active unit.

Other biological activities including neutralization and response in vivo have been indicated in preliminary experiments using material from crude amoebal extracts. Uncertainties imposed by the lack of purity were such that definitive studies in these areas have been delayed until purified NACM is available. Also, purified NACM is crucial for its complete physical characterization and determination of its range of biological activity. The assay for cytopathogenicity in cultured cells as described in this paper plays an integral part in the purification of NACM now under investigation in this laboratory.

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REFERENCES


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