The Development of a TRIC Agent (*Chlamydia trachomatis*) and its Associated Polysaccharide in Suspended Cell Cultures

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SUMMARY

The growth of large quantities of a TRIC agent and the concomitant production of polysaccharide were studied in BHK21 and L cell cultures in suspension. The maximum concentrations of elementary bodies and polysaccharide/culture were attained at about the same time (60 h) after infection in both cell systems. The yield of elementary bodies from a 1 l culture was equivalent to the maximum obtained from a total area of 8000 cm² of BHK21 monolayers. Polysaccharide found in the medium was probably derived from disrupted inclusion bodies. It is postulated that synthesis of the polysaccharide is directed by the TRIC agent but controlled by the host cell.

INTRODUCTION

Chlamydiae are obligate intracellular micro-organisms that fall into two main groups, A and B (Gordon & Quan, 1965). Members of group A are sensitive to the sulphonamides and develop within well-defined cytoplasmic inclusions that fill with a glucose polymer as infective particles form, whereas group B chlamydiae are sulphonamide-resistant and are characterized by diffuse inclusions which contain no glucose polymer.

The chlamydiae can be propagated in the yolk sac of embryonated eggs and many strains have been adapted to grow in cell culture monolayers. However, chemical and biochemical investigations have been hindered by difficulties in growing and purifying large quantities of the micro-organisms. Higashi, Tamura & Iwanaga (1962) and Tamura & Higashi (1963) overcame many of these problems by growing a chlamydia group B strain in cell cultures in suspension. The present paper describes the development of trachoma/inclusion conjunctivitis (TRIC) agent (a group A chlamydia) and the concomitant production of polysaccharide in 1 to 2 l volumes of suspension cultures of baby hamster kidney (BHK21) and L cells.

METHODS

Cell cultures. Monolayers of BHK21 cells (Stoker & Macpherson, 1964) and BHK21 cells adapted to grow in suspension (Capstick, 1963) were grown and maintained as described by Blyth, Taverne & Garrett (1971). L cells (Earle, 1943) adapted to grow in suspension were kindly supplied by Professor G. P. Manire, University of North Carolina, Chapel Hill, U.S.A. They were grown in Earle's lactalbumin hydrolysate (Gibco M11) containing 0.1 % NaHCO₃, 0.1 % yeastolate (Difco), 0.35 % glucose, 10 % (v/v) calf serum (Flow Laboratories Ltd), and streptomycin sulphate (100 µg/ml). Maintenance medium was

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growth medium without serum and also contained neomycin sulphate (25 u./ml). One to two l cultures in suspension were stirred magnetically in 4 l screw-capped bottles or 60 ml volumes were shaken in 250 ml conical flasks on an L.H.E. Mk V orbital shaker (L.H. Engineering Co. Ltd, Stoke Poges, Buckinghamshire). Growing cells were diluted with fresh medium to a concentration of about 5×10^5 cells/ml every 2 or 3 days.

TRIC agent. The fast-killing variant (Taverne, Blyth & Reeve, 1964) of strain TRIC/2/GB/MRC-4/ON (Jones, 1961) was used. Stocks of TRIC agent were partially purified from infected yolk sac material and stored and assayed as described by Taverne & Blyth (1971).

Preparation of TRIC agent inoculum for suspended cells. The concentration of yolk-sac contaminants in partially purified agent was reduced further by one passage through BHK21 monolayer cells: BHK21 monolayers were grown for 3 days in 250 ml polystyrene tissue culture flasks (Falcon Plastics Cat. no. 3024), and were infected by centrifuging on to the cells an average of two infective particles/cell (Blyth *et al.* 1971). The monolayer cell surface area was approximately 80 cm². The cells were removed in growth medium 40 to 48 h later by gently rolling glass beads over the monolayer. The cells were disrupted ultrasonically at 0 to 4 °C in a Soniprobe tank type 113/2A (Dawe Instruments Ltd) so that the inclusions were completely disintegrated. The infectivity of such preparations decreased rapidly on storage even at -70 °C, so they were used immediately after preparation, to infect cells in suspension. One bottle of infected cells usually yielded 4×10^9 to 1×10^{10} inclusion-forming units (i.f.u.), when titrated in BHK21 monolayers by the method of Taverne & Blyth (1971).

Infection of cells in suspension. L and BHK21 cells were treated identically. They were collected by centrifuging at 600 g for 10 min and resuspended in maintenance medium to a concentration of 1×10^7 cells/ml. The concentrated cells (150 to 200 ml), in a cotton-plugged 250 ml bottle, were stirred at about 100 rev./min at 36 °C with an estimated 10 to 40 infective TRIC particles/cell. At a multiplicity of infection of less than 10, fewer cells were infected; if the multiplicity of infection was 100 or more, many cells were killed. The pH of the suspension was maintained at 7.2 to 7.6 by adding 2 M-tris (tris hydroxy methyl amino methane) when required. The infected cells were stirred for 1.5 to 2 h and then poured into 1 to 2 l of fresh maintenance medium in a 4 l screw-capped bottle to give a final concentration of 1×10^6 cells/ml. They were stirred at 100 rev./min at 36 °C for the duration of the experiment.

Measurement of infectivity. Samples (10 ml) of infected suspension cultures were taken at intervals throughout the incubation and centrifuged at 600 g for 10 min. The supernatant fluids were retained for measurement of extracellular infectivity. The cells were resuspended in 2 ml growth medium and disrupted ultrasonically. The infectivity of this homogenate and of the supernatant fluid was measured in BHK21 monolayers by the method of Taverne & Blyth (1971) and expressed as i.f.u./ml of suspension culture.

Total elementary bodies. These were measured by dark-field microscopy (Reeve & Taverne, 1962).

Iodine staining of cells in suspension. Samples of infected cells were diluted with an equal vol. of Lugol's iodine. Inclusions appeared as well-defined light brown vacuoles containing particles in vigorous motion.

Measurement of polysaccharide in suspension cultures. Samples (20 ml) of the cultures were centrifuged at 600 g for 10 min. The cells were resuspended in 1 ml water and disrupted ultrasonically. Trichloroacetic acid (TCA) was added to a final concentration of 5 % (w/v). The suspensions were kept at 0 °C throughout these treatments and the TCA soluble fractions were analysed immediately or stored at -20 °C. Preliminary experiments

showed that TCA extracted all the polysaccharide from infected BHK21 cells and that it precipitated material that would otherwise have interfered with the anthrone-carbohydrate reaction. Polysaccharide in the extracts was measured by the anthrone method of Mokrasch (1954) after free glucose had been destroyed by boiling in 5 N-KOH (van der Vies, 1954). The medium, collected after removal of the cells, was also treated with TCA at a final concentration of 5 % (w/v) and centrifuged at 600 *g* for 10 min. The supernatant fluid was dialysed against at least three changes of distilled water (100 times the vol. in the dialysis bag), adjusted to pH 8 with NaOH and then concentrated on a rotary evaporator to one-tenth of its original volume. Polysaccharide was assayed as described above. Glucose standards were used and polysaccharide was expressed as glucose equivalents. Samples of suspension taken at the same time as those for polysaccharide analysis were stained with iodine and the concentration of inclusions was determined.

Enzymatic assay of polysaccharide glucose. Polysaccharide was degraded with amyloglucosidase (Sigma London Chemical Co. Ltd) and the glucose released was then measured by the glucose oxidase method (Johnson & Fusaro, 1966). The glucose oxidase mixture (Glucostat Regular, Worthington Biochemical Corp.) was reconstituted in 0.5 M-tris buffer (Dahlqvist, 1964) to inhibit the activity of carbohydrase contaminants in the mixture.

RESULTS

Yield of TRIC elementary bodies

Fig. 1 summarizes the results of three experiments in which BHK21 cells in suspension were infected with TRIC agent. In one experiment most of the cells were badly damaged 50 h after infection and representative samples of the culture could not be taken after 46 h. Cells containing inclusions seemed to be more susceptible to mechanical damage, and variation in stirring speed of the magnet may have caused disintegration of the cells. In other experiments more than 70 % of the original cells were undamaged after 48 h as judged by permeability to eosin; 20 to 30 % remained undamaged after 65 h and 1 to 8 % after 5 days. Maximum yields of elementary bodies (about 10^9 /ml of culture) were obtained 50 to 70 h after infection when 95 % of the TRIC agent was still associated with host cells. The ratio of total to infective particles was approx. one throughout the incubations.

Similar results were obtained with infected L cells in suspension (Fig. 1). The yield of cell-associated infective agent (about 10^9 i.f.u./ml of culture) was maximal 45 to 60 h after infection. The cell concentration decreased during the course of the experiment, and taking into account the L cell concentrations at particular times, the yield of infective TRIC particles/cell increased from 1.6 at 24 h to 200, 470, 680, 960 and 1400 at 30, 36, 42, 48 and 72 h respectively.

Yield of polysaccharide

Polysaccharide in BHK21 cells was first detected by the anthrone-sulphuric acid test 24 h after infection. Infectivity and the amount of polysaccharide/culture reached their maximum values at about the same times (Fig. 1, 2). Inclusions in BHK21 cells first reacted with iodine about 24 h after infection; thereafter they stained progressively more deeply and increased in size until by 48 h they occupied most of the cytoplasm. Those few cells that remained whole 5 days after infection were distorted by enormous inclusions. Inclusions in L cells often stained poorly with iodine and were then recognizable only by their characteristic shape and the elementary bodies they contained. Glucose released by amyloglucosidase accounted for 90 to 94 % of the polysaccharide isolated from infected BHK21 cells.

Fig. 1. The yield of infective TRIC agent from suspended cells. Results for BHK21 cells are from three experiments and show agent associated with cells (—○—) and released into medium (—●—); the results for L cells (---△---) are for cell associated agent only.

Fig. 2. Polysaccharide in TRIC-infected suspended cell cultures. Cells and medium were separated by centrifugation at 600g for 10 min. Polysaccharide in TCA extracts was measured by the anthrone method and expressed as glucose equivalents. □, ○, BHK21 cells (two separate experiments); △, L cells; ●, BHK21 cell medium.

Fig. 3. Polysaccharide in TRIC-infected suspended cells. The results are expressed as glucose equivalents/inclusion. O, □, ×, BHK21 cells; △, L cells.

Approximately 20 % of the total polysaccharide found in the BHK21 cultures at 48 h was free in the medium; this proportion increased, probably as a result of cell disruption, as incubation continued. As expected from microscopy of iodine-stained cells, the amount of polysaccharide/inclusion increased throughout the whole incubation period (Fig. 3). Polysaccharide in homogenates of infected cells was rapidly degraded unless enzymatic activity was inhibited, and the decrease in total polysaccharide content of the culture was probably due to amylolytic activity within damaged cells or in the medium.

The polysaccharide content of infected L cells was less than that of infected BHK21 cells in the experiments depicted in Fig. 3. This was not invariably so; yields of polysaccharide varied from batch to batch but the reason for this is unknown. When a single batch of infected L cells was used, increasing the glucose concentration of the maintenance medium to twice or three times the normal (0.35 %) did not significantly alter the amount of polysaccharide produced.

DISCUSSION

The methods described for the production of large quantities of TRIC agent could provide sufficient starting material for detailed analyses of the infective elementary bodies and the more actively metabolic forms of TRIC agent, the reticulate bodies. The multiplicity of infection (10 to 40) was chosen to give the highest number of infected cells and the greatest yield of elementary bodies per culture; under these conditions, the yield (about 10^{12} i.f.u.) from 1 l of culture containing 10^9 infected BHK21 or L cells was equivalent to the maximum that would be obtained from a total surface area of 8000 cm² of BHK21 monolayer cells (i.e. the yield from one hundred 250 ml Falcon Plastics flasks). The yield and ratio of infective to total elementary bodies were similar to those from infected monolayer cells (Evans, 1972). Provided that most of the infected cells remained whole the yield of elementary bodies/ml of culture was greatest about 60 h after infection.

The exact composition and structure of the polysaccharide in the inclusions of group A chlamydiae are unknown. On the basis of its reaction with iodine and its digestion by salivary amylase (Rice, 1936) the polysaccharide has been referred to as glycogen. Our results support this conclusion. However, although glucose was the only sugar found by chromatography of acid hydrolysates of polysaccharide extracted from BHK21 cells (A. J. Garrett, to be published), an assay using amyloglucosidase and glucose oxidase indicated that either 5 to 10 % of the polysaccharide was not glucose or it was not glucose bound as in glycogen.

The site and mode of synthesis of this polymer have not been clearly demonstrated. When several batches of suspended cells were examined 48 h after infection with TRIC agent, there was no correlation between the yields of polysaccharide and the number of infective organisms that each cell in a batch had received, or between the yields of polysaccharide and infective elementary bodies/inclusion remaining at any one time. It might be concluded, therefore, that the host cell, not the micro-organism, synthesized the polysaccharide. However, recent evidence from Jenkin & Fan (1971) and A. Evans (personal communication) suggests that the micro-organism itself produces an enzyme (or enzymes) that directs polysaccharide synthesis in TRIC-infected cells. Jenkin & Fan (1971) demonstrated that homogenates of TRIC-infected HeLa cells incorporated [^{14}C]glucose from ADP-[^{14}C]glucose into a product that co-precipitated with shellfish glycogen. ADP-glucose was used preferentially by the infected cells and UDP-glucose by normal HeLa cells. These results imply the presence of an enzyme coded for by the micro-organism but it was not possible to determine whether micro-organism or host synthesized the enzyme. Evans measured the amount of polysaccharide produced 48 h after infection of BHK21 cells with TRIC agent. She showed that polysaccharide production was inhibited in the presence of chloramphenicol which was added within 24 h of infecting the cells, at a concentration that inhibits bacterial but not mammalian protein synthesis. This suggests that the micro-organism synthesizes the enzyme system responsible for polysaccharide formation.

When these results are considered together, we may postulate that although the polysaccharide produced by TRIC-infected cells is synthesized by enzymes from the micro-organism, the host cell controls the amount of polymer formed.

As in TRIC infections of monolayer cultures (Reeve & Taverne, 1967; Evans, 1972), polysaccharide production in suspended cells accompanied the development of elementary bodies. Although the maximum polysaccharide concentration/culture was reached 50 to 60 h after infection of suspended cells, the average polysaccharide content of inclusions continued to increase for at least 30 h after this (Fig. 3). This apparent anomaly resulted from disruption of some inclusions and the subsequent enzymatic degradation of polysaccharide during the incubation. Reeve & Taverne (1967) and Evans (1972) showed that the average polysaccharide content of inclusions in BHK21 monolayer cells decreased rapidly after attaining its maximum 48 h after infection; the decrease was attributed to leakage from the cells and not to their rupture. In suspended cells the amount of polysaccharide released from disrupted inclusions could account for that found in the medium. Inclusions in undamaged suspended cells increased in size, polysaccharide content and intensity of staining with iodine for at least 90 h, so if leakage of polysaccharide occurred it was more than compensated for by synthesis within the inclusion. Polysaccharide in TRIC-infected suspended cells may well be retained more efficiently than that in monolayer cultures; BHK21 cells spread on glass are permeable to eosin, whereas cells grown in the same way and removed from the glass support with EDTA and trypsin are impermeable to the dye (A. Evans, personal communication).

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REFERENCES

- BLYTH, W. A., TAVERNE, J. & GARRETT, A. J. (1971). Trachoma inclusions separated from cells. In *Trachoma and Related Disorders Caused by Chlamydial Agents*. Proceedings of a Symposium held in Boston, Massachusetts, pp. 79–87. Edited by R. L. Nichols. Amsterdam: Excerpta Medica.
- CAPSTICK, P. B. (1963). Growth of baby hamster kidney cells in suspension. *Proceedings of the Royal Society of Medicine* **56**, 1062–1064.
- DAHLQVIST, A. (1964). Method for assay of intestinal disaccharidases. *Analytical Biochemistry* **7**, 18–25.
- EARLE, W. R. (1943). Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cell. *Journal of the National Cancer Institute* **4**, 165–212.
- EVANS, A. (1972). The development of TRIC organisms in cell cultures during multiple infection. *Journal of Hygiene* **70**, 39–48.
- GORDON, F. B. & QUAN, A. L. (1965). Occurrence of glycogen in inclusions of the psittacosis–lymphogranuloma venereum–trachoma agents. *Journal of Infectious Diseases* **115**, 186–196.
- HIGASHI, N., TAMURA, A. & IWANAGA, M. (1962). Developmental cycle and reproductive mechanism of the meningopneumonitis virus in strain L cells. *Annals of the New York Academy of Sciences* **98**, 100–121.
- JENKIN, H. M. & FAN, V. S. C. (1971). Contrast of glycogenesis of *Chlamydia trachomatis* and *Chlamydia psittaci* strains in HeLa cells. In *Trachoma and Related Disorders Caused by Chlamydial Agents*. Proceedings of a Symposium held in Boston, Massachusetts, pp. 52–59. Edited by R. L. Nichols. Amsterdam: Excerpta Medica.
- JOHNSON, J. A. & FUSARO, R. M. (1966). The quantitative enzymic determination of animal liver glycogen. *Analytical Biochemistry* **15**, 140–149.
- JONES, B. R. (1961). TRIC virus infections in London. *Transactions of the Ophthalmological Society of the United Kingdom* **81**, 367–378.
- MOKRASCH, L. C. (1954). Analysis of hexose phosphates and sugar mixtures with the anthrone reagent. *Journal of Biological Chemistry* **208**, 55–59.
- REEVE, P. & TAVERNE, J. (1962). A simple method for total particle counts of trachoma and inclusion blennorrhoea viruses. *Nature, London* **195**, 923–924.
- REEVE, P. & TAVERNE, J. (1967). Strain differences in the behaviour of TRIC agents in cell cultures. *American Journal of Ophthalmology* **63**, 1167–1173.
- RICE, C. E. (1936). The carbohydrate matrix of the epithelial-cell inclusion in trachoma. *American Journal of Ophthalmology* **19**, 1–8.
- STOKER, M. & MACPHERSON, I. (1964). Syrian hamster fibroblast cell line BHK21 and its derivatives. *Nature, London* **203**, 1355–1357.
- TAMURA, A. & HIGASHI, N. (1963). Purification and chemical composition of meningopneumonitis virus. *Virology* **20**, 596–604.
- TAVERNE, J., BLYTH, W. A. & REEVE, P. (1964). Toxicity of the agents of trachoma and inclusion conjunctivitis. *Journal of General Microbiology* **37**, 271–275.
- TAVERNE, J. & BLYTH, W. A. (1971). Interactions between trachoma organisms and macrophages. In *Trachoma and related disorders caused by chlamydial agents*. Proceedings of a Symposium held in Boston, Massachusetts, pp. 88–107. Edited by R. L. Nichols. Amsterdam: Excerpta Medica.
- VAN DER VIES, J. (1954). Two methods for the determination of glycogen in liver. *Biochemical Journal* **57**, 410–416.