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Some consequences of the multiple infection of cell cultures by TRIC organisms

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SUMMARY

In BHK 21 cells infected with more than one TRIC organism the inclusions coalesce so that 30 hr. after infection only one inclusion per cell remains. Six hours after infection the cells are as susceptible to further infection as uninfected cells.

INTRODUCTION

In conjunctival smears from patients with trachoma the proportion of cells that contain specific inclusions is usually small. It is therefore not surprising that cells containing more than one inclusion are rare; the number of infective organisms free at any one time would make multiple infection unlikely. Experimentally, cell cultures can be infected with a large number of trachoma organisms so that high multiplicities of infection can be achieved. Nevertheless, at the end of the growth cycle only one inclusion per cell is usually seen; it could arise from the union of several inclusions or from the development of the first organism to enter the cell, with the subsequent exclusion or inhibition of all others. The results of experiments done to distinguish between these possibilities are reported here.

METHODS

TRIC agents. The following strains were used; the suffix *f* denotes fast-killing variants (Taverne, Blyth & Reeve, 1964). The abbreviations used in this paper are given in parentheses.

TRIC/2/China/Peking-2/OT*f* (T'ang, Chang, Huang & Wang, 1957) (PK 2*f*).

TRIC/2/SAU/HAR-2/OT*f* (Murray *et al.* 1960) (SA 2*f*).

Preparation of pools. Pools were made from infected yolk sacs; they were treated with KCl and stored in 0·25 M sucrose in buffer as previously described (Taverne & Blyth, 1971).

Cell culture methods and media have been described (Taverne & Blyth, 1971).

Inclusion counts were made after centrifuging TRIC agents onto monolayer cultures of BHK 21 cells seeded 24 hr. before. Cultures were fixed with methyl alcohol and stained with Giemsa.

RESULTS

Cells infected with more than one organism and examined within 20 hr. of infection often contain more than one inclusion, although by 30 hr. there is rarely more than one inclusion per cell. Thus more than one organism must be able to enter a cell within the short period allowed for infection and, in these circumstances, at least the early stages of replication proceed normally. To investigate the apparent disappearance of inclusions, cell cultures were infected with 15 inclusion-forming units of strain PK 2*f* per cell and examined microscopically during the period 18–28 hr. after infection. Cells containing inclusions apparently in the process of coalescence were repeatedly observed (Plate 1). The time course of coalescence of inclusions was therefore determined by counting the inclusions per cell at various times (Table 1).

Inclusion counts could not be made with confidence earlier than 17 hr. after infection. At this time there was an average of six, indicating that some had already coalesced. From 17 to 24 hr. the number decreased progressively, until by 28 hr. no cells were observed with more than one inclusion. When many inclusions were grouped closely and were coalescing, it was sometimes difficult to decide whether they were still separated. Errors resulting from this difficulty would lead to an underestimate of the true number. After coalescence inclusions appeared larger and at a more advanced stage of development than those of equivalent age resulting from a single infection.

In the experiment just described all the infecting organisms must have been adsorbed by the cell within the 30 min. period of centrifugation. To determine whether the presence of a developing inclusion in a cell alters the chance of later infection, it was necessary first to define the time interval that would allow inclusions of different ages to be differentiated most clearly, and then to choose the time after infection most suitable for recognizing such inclusions. The initial bodies in inclusions become intermediate bodies and elementary bodies between 18 and 24 hr. after infection. Furthermore, during this time the diameter of inclusions resulting from infection with one organism almost doubled, so that inclusions of these ages could easily be distinguished.

To investigate the possibility that the presence of an inclusion changed the susceptibility of a cell to subsequent infection, cultures were first centrifuged with a concentration of organisms of strain SA 2*f* sufficient to infect most of the

Table 1. *The number of inclusions seen in BHK cells at various times after infection with* 15 *inclusion-forming units per cell of strain PK* 2*f*

Hour after infection	No. cells counted	Mean no. inclusions per infected cell	Range	± Standard error
17	67	6·1	2–22	0·38
18	49	4·4	2–11	0·32
20	71	3·2	1–9	0·23
24	60	1·8	1–7	0·16
28	> 100	1	1	—

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cells, but such that there was less than a 1% chance of multiple infection. Six hours later the cultures were again inoculated by centrifugation; they were fixed 24 hr. after the end of the first centrifugation, together with controls that had been infected only at one or other time (Table 2). Within each culture, counts were made in the same microscope fields, selected at random, of the cells that contained both 18 and 24 hr. inclusions (Plate 2), and of the number singly or multiply infected with inclusions of either age. The total number of cells in each field was also counted. Thus the chance of any cell becoming infected in either or both centrifugations could be calculated. The proportions of cells with 18 or 24 hr. inclusions in control cultures infected only at one or other time tallied with those in the doubly infected cultures.

Were the entry and development of new organisms not altered in infected cells, the chance of an infected cell supporting the multiplication of a second organism would be the same as that for any cell in the culture. Thus, in the first culture the chance of an inclusion developing within a cell after the first centrifugation was $\frac{64}{632}$. The chance of a second inclusion developing in a cell already bearing an inclusion was thus $\frac{64}{632} \times \frac{152}{632}$. In the 632 cells counted, the number of cells with an inclusion from both infections was predicted to be $\frac{64}{632} \times \frac{152}{632} \times 632 = 15$. This figure, and those predicted for the other three cultures, agrees well with the observed result, suggesting that our original assumption was correct.

DISCUSSION

The results reported here extend the observation made by Bernkopf, Mashiah & Becker (1962) and Kramer & Gordon (1971) that TRIC inclusions within a cell at first develop separately and eventually coalesce. Inclusions tend to aggregate in the area of cytoplasm near the nucleus, but contact is apparently not in itself sufficient to cause immediate coalescence since early inclusions frequently appeared to be touching each other but remained discrete.

A conclusion of practical importance is that in infectivity titrations based on inclusion counts in cell cultures spuriously low results can be obtained if multiple infection is not rigorously excluded.

As inclusions cannot be counted accurately earlier than 17 hr. after infection,

Table 2. *The number of cells containing inclusions in cultures inoculated at* 18 *and again at* 24 *hr with strain SA 2f*

	Cells with 18 hr. inclusions		Cells with 24 hr. inclusions		
No. of cells examined	Total	Cells with > 1 18 hr. inclusion	Total	Cells with 18 hr. inclusion in addition	Predicted no. cells with both 18 hr. and 24 hr. inclusions
632	152	21	64	18	15
592	88	12	78	10	12
335	114	33	60	19	20
299	51	6	48	9	9

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the question whether coalescence occurred only after some hours of development or proceeded at a constant rate from the time of infection could not be answered directly. Statistical analysis of the numbers of inclusions present in infected cells at different times showed that the values fitted curves representing either possibility, and that the chances of one being a better fit than the other were not significantly different.

In the experiments with cultures infected twice, there was obviously a chance that within any cell an 18 and 24 hr. inclusion might coalesce. The degree to which such coalescence affected the results could not be calculated from the earlier experiment since there the multiplicity of infection was much higher, i.e. 15 organisms per cell as opposed to less than one. Nevertheless, the good agreement between the predicted and observed numbers of doubly infected cells suggests that such coalescence was not a significant factor.

The second infection of cells already bearing inclusions indicated that their surface was not altered in a way that excluded entry of another organism. Similarly, although TRIC organisms induce the formation of interferon and are susceptible to its action (Hanna, Merigan & Jawetz, 1967), any interferon present 6 hr. after infection did not prevent the apparently normal development of further inclusions.

The question has been raised as to how readily the conjunctiva of an individual already suffering from trachoma can be re-infected (Nichols, von Fritzinger & McComb, 1971). Our experiments, done in conditions where neither host defence mechanisms nor epidemiological factors operate, show that individual infected cells are completely susceptible to infection by organisms of the same strain at least for a few hours after entry of the first organism.

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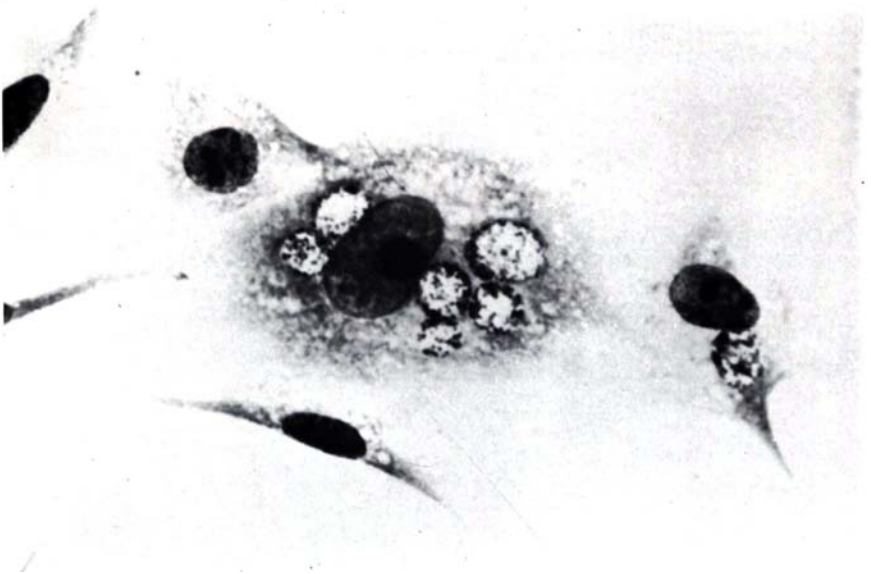

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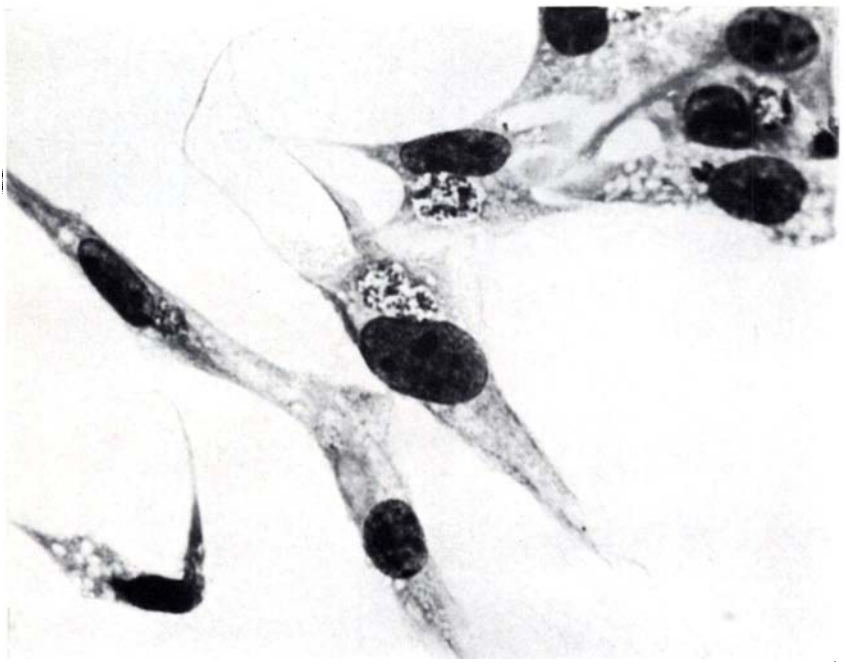

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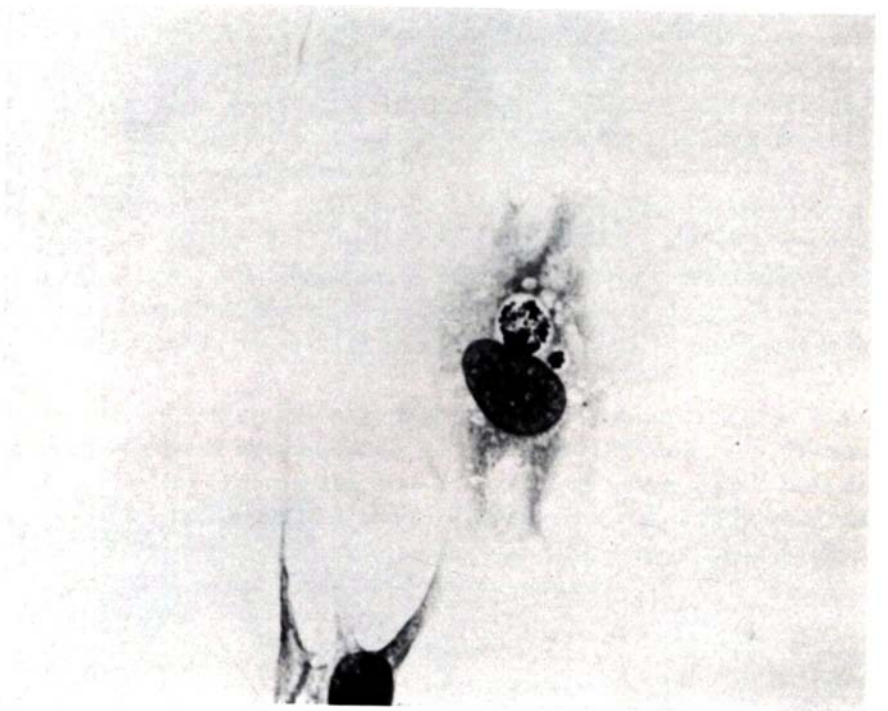

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

BHK cells multiply infected with strain PK 2*f* 24 hr. earlier; (A) shows a cell with six discrete inclusions, (B) shows one with two inclusions that have apparently just coalesced.

PLATE 2

A BHK cell with two inclusions. The culture was infected twice with strain SA 2*f*, 24 and 18 hr. earlier. The inclusions resulting from each infection can readily be identified.