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Nuclear Polyhedrosis Virus Detection: Relative Capabilities of Clones Developed from *Trichoplusia ni* Ovarian Cell Line TN-368 to Serve as Indicator Cells in a Plaque Assay

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Cloned cell lines from the established *Trichoplusia ni* line TN-368 appear to differ from one another in their relative capabilities to serve as plaque assay indicator cell lines for *Autographa californica* nuclear polyhedrosis virus. Although there seems to be little correlation between their relative generation times and their efficiency in supporting plaque formation as indicator cell lines, there does seem to be a relationship within a given line between its capability to serve as an indicator and its phase of growth as a population; i.e., lag, logarithmic, or stationary. Both the parent line and clone 10 were more efficient indicators when they were in the logarithmic phase of growth than when in either the lag or stationary phases. Also, there appears to be a rough correlation between the capability of a given clone to serve as an indicator and the rate at which polyhedra first appear in the nuclei of the infected cells, with the best indicators producing polyhedra first. Increased incubation time has no effect on equalizing the plaque assay results for the less efficient clones. It was observed, also, that those clones that are the least efficient as plaque assay indicators produce the most external PFU per cell.

The study of Lepidopteran nuclear polyhedrosis viruses (NPV) was greatly facilitated by the establishment of *Trichoplusia ni* ovarian cells in continuous culture (3), as well as by the subsequent development of a plaque assay using these cells for infectious NPV detection (4). However beneficial the advent of this plaque assay was, we were concerned about its degree of fidelity. One reason for this concern was that the cell line used as the indicator line was uncloned and, possibly, might not be as sensitive an indicator as is desirable because of its potential genetic heterogeneity. Another reason was that the plaque assay is dependent upon the production of polyhedra by infected cells, both the cells infected primarily and the adjacent cells infected secondarily. Although it is commonly accepted that cells infected by NPV produce polyhedra, there is no rigorous proof to show that this is always the case. The production of polyhedra may well require different intracellular conditions than the production of infectious virus, and thus polyhedra production may not be the most sensitive indicator of infection. Knudson and Tinsley (5) have suggested that *Spodoptera frugiperda* cells grown to confluency are incapable of producing cytopathic effect (CPE) upon NPV infection. Further, James Vaughn observed that a special cell line of *Porthetria dispar* infected with *P. dispar* NPV showed no signs of CPE, yet when these cells were examined with an electron microscope numerous intranuclear enveloped viral nucleocapsids were seen (personal communication).

We decided, therefore, to investigate some of the aspects of the assay beginning by cloning the TN-368 line and comparing the cloned daughter lines with regard to their relative capabilities to serve as indicator cells in the plaque assay. In addition, we examined the relative rates of CPE appearance among the infected clones, as well as the relative amounts of infectious extracellular particles they produced.

MATERIALS AND METHODS

Cells. The parent cell line TN-368 was originally established in continous culture from the ovaries of newly emerged, virgin female moths (3). Subsequently, daughter cell lines were developed from isolated single cells (L. E. Volkman and M. D. Summers, *in Proceedings of the IV International Conference on Invertebrate Tissue Culture*, in press), and these cloned lines and TN-368 have been maintained in medium TNM-FH (3). The cells were subcultured routinely every 3 days by merely pipetting the loosely adhering cells several times to make a uniform suspension and then transferring them to a

new flask. Plastic Falcon tissue culture flasks (25 ml) were seeded with 10^5 to 2×10^5 cells/ml in 6 ml of medium.

Autographa californica inoculum. The inoculum, kindly supplied by Pat Vail, was the supernatant fluid of a $1,000 \times g$ for 15-min centrifugation of 5-day-old infected TN-368 cells. The cells were infected with *T. ni* hemolymph containing *Autographa californica* nuclear polyhedrosis virus.

Plaque method for the assay of infectivity. The method used for determining infectivity was a slightly modified version of the plaque assay procedure reported by Hink and Vail (4). Corning 35-mm tissue culture dishes (no. 2500) were each seeded with 3.5×10^5 cells in 2 ml of medium. The indicator cells were growing in logarithmic phase and were between the concentrations of 2×10^5 and 6×10^5 cells/ml when used. The cells were dispersed homogeneously in the dish and were incubated for 1 to 3 h at 28 C, during which time the cells attached to the bottom of the dish. The medium was then pipetted off, and 0.1 ml of virus suspension was added to the center of each dish. The inoculum was allowed to spread from the center of the dish outward, and after 1 to 2 min the dishes were picked up in stacks of three and were tilted about 75° from horizontal by hand. They were held there for approximately 4 s and then were tilted in the opposite direction in the same manner and held there for 4 s. This oscillatory action was repeated for five to six cycles. The dishes were then rotated 90° and the same action was repeated. This movement allowed a thin layer of inoculum to flow over the cell surfaces. This rocking procedure was repeated every 15 min for 1 h. After the 1 h adsorption period, a 2-ml overlay of 0.9% methylcellulose in TNM-FH medium was applied to the internal periphery of each dish. The dishes were placed on plastic trays and inserted into plastic bags for a 72-h incubation period at 28 C. After that period, the methylcellulose layer was carefully removed, and the plaques were counted with the dishes right side up on a stereoscopic microscope illuminated by a Bausch & Lomb model PR-27 research illuminator.

Assays were routinely done in triplicate. The standard deviations were rarely more than 20% of the mean and never more than 25%.

Synchronous infection. The synchronous infection experiments were done with the same cell populations that were used as indicator cells in coincident plaque assays. For each experiment, 2×10^6 cells were transferred to new 25-ml Falcon tissue culture flasks where they were held at 28 C for approximately 2 h while the cells attached to the plastic. The medium was then removed, and 1 ml of inoculum containing 10×10^6 PFU (when assayed on TN-368 cells) was added. This amount of virus, at a multiplicity of infection of 5, is the amount of virus predicted by the Poisson distribution needed to insure infection of more than 99% of the cells. The virus was allowed to adsorb for 1 h while the flasks were being rocked slowly on a Bellco rocker platform at a setting of 4. After this period, 4 ml of fresh medium was added to each flask, and the flasks were incubated at 28 C. Samples were withdrawn periodically, the cells were pelleted by centrifugation at $300 \times g$ for 15 min, and the supernatant fluid was removed and frozen at −40 C for later titration by the plaque assay method using TN-368 cells. The cells in the pellets were resuspended, and 300 to 400 were inspected with a Zeiss phase microscope for signs of CPE.

RESULTS

Effects of phase of growth on plaque assay indicator cells. Cells taken from the lag, logarithmic, and stationary phases were compared as indicator cells in the plaque assay (Fig. 1). In the case of both TN-368 and clone 10, two to three times more plaques were apparent when using log-phase cells than when using the lag-phase cells, and approximately two times more appeared with log phase than with stationary phase.

In view of these results, all plaque assays were done with logarithmic-phase cells.

Comparison of the cloned lines and TN-368 as indicator cells in the plaque assay. The cloned lines were compared with TN-368

PFU per plate

	lag	log	stationary
TN 368	38	95	67
Clone 10	41	101	47

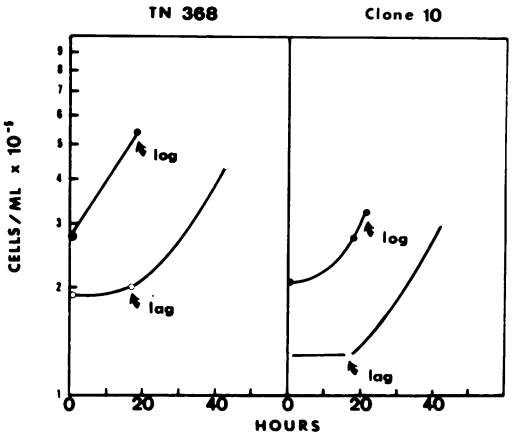

FIG. 1. *Dependence of plaque assay results on stage of indicator cell growth. Cells from both clone 10 and TN-368 in lag, logarithmic, and stationary phases of growth were compared as indicator cells in the plaque assay. (Arrows indicate the time when the cells in lag and logarithmic phases were taken for use in the plaque assay. Stationary cells used were from 100-h-old cultures.) The conditions for the assay are described in the text. An identical inoculum was used on all the plates.*

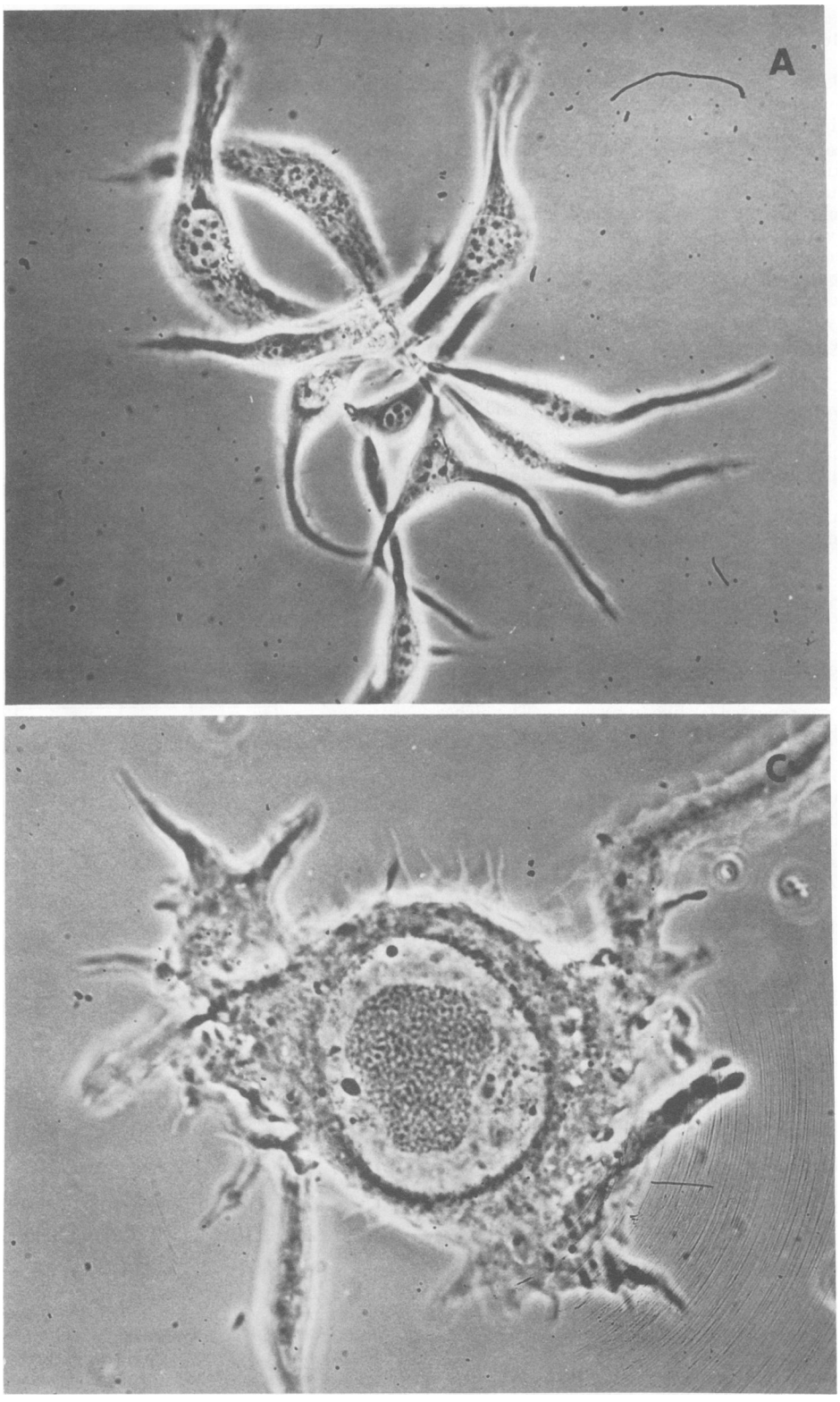

FIG. 2. *Progression of CPE induced in T. ni ovarian cells by A. californica nuclear polyhedrosis virus. (A) Uninfected clone 3 cells (×3,200). (B) First stage of CPE. The cell has rounded up, the nucleus is enlarged, all nuclei have disappeared, and the virogenic stroma has formed (×8,000). (C) Virogenic stroma seems to have become more compact. Often there is Brownian motion around the internal periphery of the nuclear membrane during this stage (×8,000). (D) Cells containing polyhedra (×8,000).*

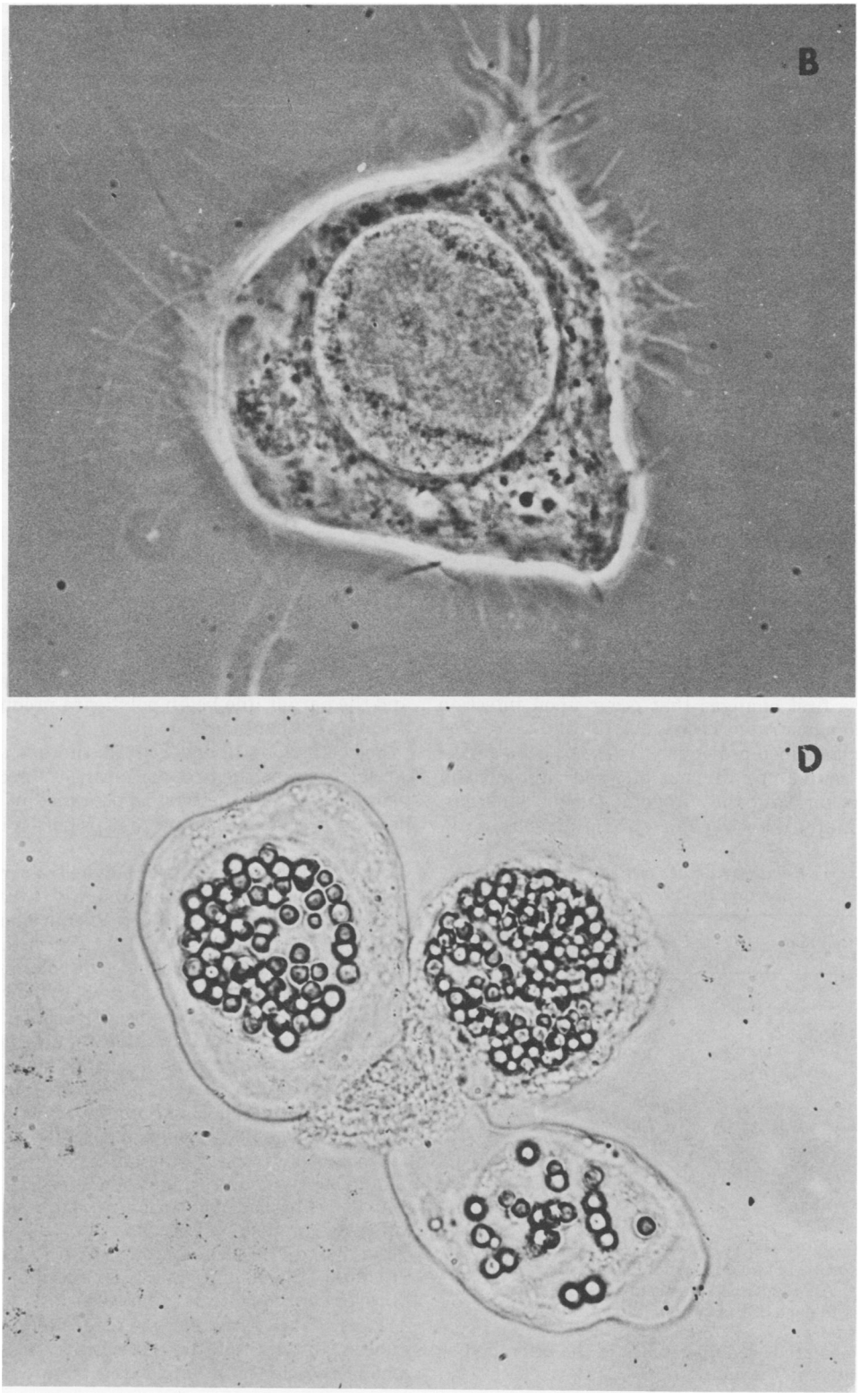

FIG. 2

with regard to their capability to serve as indicator cells in the plaque assay.

Table 1 shows the relative number of plaques that appears on the various indicator lines when they are infected with an identical inoculum. The parent line and clone 10 are the best indicators, giving the highest number of plaques, followed by clones 3 and 8 and then by 5 and 13. It is of interest to note that the relative capability of a line to serve as an indicator seems to be independent of the generation time. That is, for example, even though the population doubling time of clone 13 was as short or shorter than that of either TN-368 or clone 10, fewer plaques appeared. These results indicate that the cells of some clones are less receptive to infection than the cells of other clones or that a larger proportion of infected cells in some clones produce polyhedra less readily or both.

Synchronous infection. Synchronous infection experiments were done using cells from the same populations that were used as indicator cells in coincident plaque assays.

It was expected that data on the relative extent of infection of the clones with a higher multiplicity of infection, as well as the relative rates of appearance of both prepolyhedral and polyhedral CPE, might aid in explaining their differing plaque-forming capabilities.

The first changes that occur upon infection are demonstrated in Fig. 2A, B, and C. As has been described previously (1, 7), the cells generally round up, the nuclei swell, all nucleoli disappear, and the virogenic stroma emerges. Gradually the virogenic stroma appears to diminish slightly and become more compact. Often there is Brownian motion between the stroma and the nuclear membrane. These morphological changes will be referred to in this paper as prepolyhedral CPE. Figure 2D illustrates the polyhedral stage of CPE that occurs later.

TABLE 1. *Relative number of PFU revealed by TN-368 and daughter cell lines*

Cell line	PFU/plate[a]	Generation time (h)[b]
10	280	17
	269	14
TN-368	264	18
	264	16
8	156	16
	213	15
3	190	16
	196	16
5	64	16
	52	17
13	46	14
	85	15

[a] Results of two experiments, each assayed in triplicate. An identical inoculum was used on all the plates in each of the assays. Assay conditions are in the text.

[b] Population doubling times of the cells used as indicators in the assays.

A comparison of the appearance of total CPE among the clones is shown in Fig. 3. Total CPE is defined here as the sum of both the prepolyhedra-containing cells and the polyhedra-containing cells per total cells viewed. As indicated in the graph, both the parent line and clone 10, the highest capable indicators in the plaque assay, begin to show CPE at about 10.5 h postinfection, whereas the other lines start from 1.5 to 3.5 h later. The rates of development of total CPE, once it starts to appear, are all about the same, with those of clones 13 and 5 being just slightly less. By 36 h (although not indicated in Fig. 3), 100% of the cells in all the lines show CPE.

The relative rates of appearance of polyhedra are shown in the right-hand portion of Fig. 3. It should be noted that in all cases the relative rates of appearance of polyhedra are less than the rates of appearance of total CPE. This may indicate that a rate-limiting step occurs for crystal maturation. As might be expected, clones 10 and the parent line begin polyhedra production before the other clones do.

For both clone 10 and TN-368, there is a 7- to 8-h lag between the first appearance of prepolyhedral CPE and the first appearance of polyhedra. For clones 8, 5, and 13, this period is 9 to 10 h.

At 24 h, the percentage of polyhedra-containing cells in each of the clones roughly correlates with the plaque assay values in that clone 10 and TN-368 contain the highest percentage of polyhedra and are about equal, with clones 8, 3, 5, and 13 containing less. By 48 h, however, these latter values increase considerably.

Plaque assay with extended incubation. In view of the above results, it seemed that those clones that did relatively poorly as indicator cells in the plaque assay might reveal more plaques with a lengthened incubation period. Table 2 shows the results of a comparative plaque assay incubated for 96 h. It can be seen that the extended incubation period makes no difference, and that, just as in the 72-h assay, clones 8 and 3 still exhibit only 60 to 70% of the PFU that TN-368 and clone 10 do and that 5 and 13 reveal only about 25 to 30%.

Titers from synchronous infection experiments. In view of the differing capabilities among the clones of supporting plaque forma-

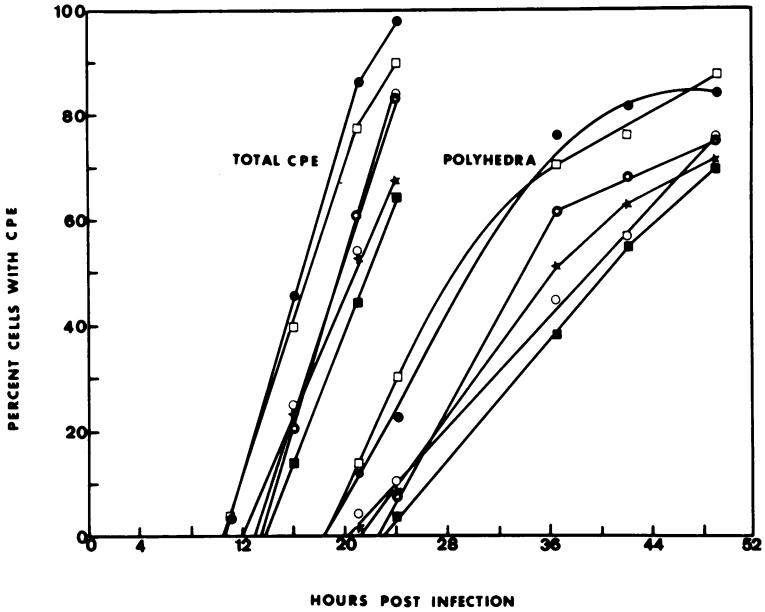

FIG. 3. *Rate of appearance of CPE among the cloned lines and TN-368 synchronously infected with A. californica NPV. At various times, 300 to 400 cells were viewed microscopically for signs of CPE, both prepolyhedral and polyhedral. "Total CPE" is the sum of both. By 36 h, although not indicated in the graph, 100% of the cells in all the lines showed CPE. The conditions of infection are described in the text. Symbols: □, TN-368; ●, clone 10; ★, clone 8; ○, clone 3; ★, clone 5; ■, clone 13.*

TABLE 2. *Effect of extended incubation on plaque assay results*

Indicator cell line	Ratio[a]	
	72 h[b]	96 h
Clone 10	1.03	1.04
Clone 8	0.70	0.69
Clone 3	0.72	0.60
Clone 5	0.24	0.27
Clone 13	0.25	0.33

[a] Results expressed as the ratio of the average PFU per plate obtained when using each of the cloned lines as the indicator line relative to the average PFU per plate obtained with TN-368 cells.
[b] Incubation period.

tion (i.e., polyhedra production), it was of interest to determine whether there were differences among them in producing extracellular infectious particles. Table 3 presents the results of samples assayed for extracellular infectious particle content that were removed at 24, 48, and 72 h during the synchronous infection experiments. The data suggest, among other things, that the extracellular PFU exhibit some loss of infectivity at 28 C. In several cases, the 72-h PFU value is less than the 48-h value. This point was further explored, as reported in the following section. The data also indicate that the greatest PFU per cell values are obtained when the cell population from which the samples are taken contain between 70 and 90% cells with polyhedra. This occurs between 24 and 48 h postinfection for clone 10 and TN-368 and between 48 and 72 h postinfection for clones 3, 8, 5, and 13. Furthermore, the data show that clones 5 and 13, the least-sensitive plaque assay indicators, produce the most extracellular PFU per cell.

Loss of extracellular PFU infectivity at 28 C and 4 C. Figure 4 shows the loss in titer that results from holding extracellular PFU grown in clone 10 and clone 13 cells at 4 C and 28 C for extended periods of time. The titer of the unfrozen virus grown in clone 10 cells was 1.2×10^8 PFU/ml, whereas that of clone 13 was 1.49×10^8 PFU/ml, thus reflecting again the greater extracellular PFU production by clone 13. The virus samples were frozen at −90 C and, upon their subsequent thawing 9 days later, the titer of the clone 10 virus had dropped to 6.4×10^7 PFU/ml (47% loss), and the clone 13 virus had dropped to 1.13×10^8 (25% loss).

Figure 4 shows that, in the case of both the fresh and the frozen virus grown in clone 10 and 13 alike, the loss of infectivity of the extracellular PFU is greater at 28 C than at 4 C, with the major portion of the loss occurring in the first 10 h. In each case, at least 30% of the virus activity was lost in the first 10 h when it was held at 28 C.

TABLE 3. *Extracellular PFU[a] per cell at 24, 48, and 72 h*

Cell line	Avg PFU/cell			Polyhedra-containing cells (%)		Avg of highest values of avg PFU/cell
	t = 24 h	t = 48 h	t = 72 h	24 h	48 h	
TN-368	70[b]	48[b]	47[b]	72[c]	93[c]	71
	49	72	56	30	87	
10	75	61	33	78	88	84
	48	92	58	22	84	
3	78	76	55	36	74	83
	40	90	67	10	75	
8	36	57	35	38	73	74
	18	53	90	8	75	
5	51	97	60	24	86	110
	25	109	123	8	71	
13	74	81	31	34	71	129
	42	177	166	4	69	

[a] Results of two experiments. All samples were processed as described for the synchronous infection experiments in the text. PFU determinations were done using TN-368 cells as the indicator.

[b] Calculated by subtracting the average PFU per milliliter at t = 0 from the average PFU per milliliter of t = x and dividing by the cell concentration at t = 0.

[c] Determined by inspecting 300 to 400 cells with a phase microscope.

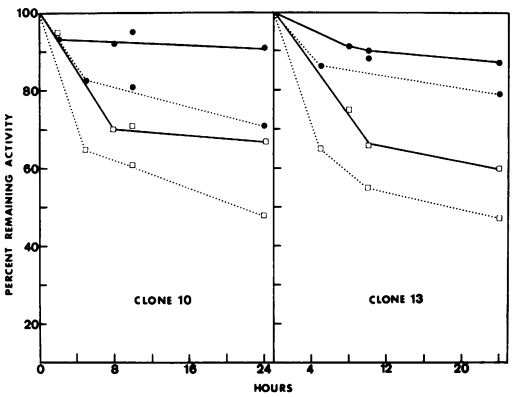

FIG. 4. *Clone 10 and clone 13 cells were synchronously infected as described in the text. The culture medium was collected when 70 to 90% of the infected cells contained polyhedra. The cells were removed by centrifugation, and the appropriate dilutions of the supernatant fluid were made and held at 4 C (●) and 28 C (□) for assay at subsequent intervals (——). A second portion of the supernatant fluid was frozen immediately at −90 C for 9 days, after which time it was thawed and assayed as above (– – –).*

DISCUSSION

T. ni ovarian cell line TN-368 has routinely been used as the indicator cell line in a plaque assay developed for the quantitation of Lepidopteran nuclear polyhedrosis virus (4). This cell line is uncloned and, in the interest of attempting to reduce the variability of the results and increasing the efficiency of the assay, several cloned lines derived from TN-368 were developed and tested for, among other things, their capability to serve as indicator cells in the plaque assay. It was found that the cloned lines did, indeed, differ from one another and from the parent line in this capability. Most of the clones were less efficient than TN-368 as plaque assay indicators. Clone 10, however, proved to be at least equally, and perhaps slightly more, efficient.

The reason for these differences in indicator capabilities may be the obvious one, that in fact some cloned lines are less susceptible to infection than others; or, perhaps more obscurely, the differences might lie in the mechanics of polyhedra production. These differences cannot be attributed to rates of population growth and cell division since the generation times for the cloned lines are all very similar. The time required for external infectious particle production may be involved since the lines that are the best indicators produce polyhedra before the others do, and they may well produce external particles more rapidly as well. Since the infection must spread to adjacent cells for the plaques to become visible and those adjacent cells must be in log phase to develop CPE most reliably, timing could be a critical factor in this assay. Experiments are in progress to test this idea.

Evidence (CPE) was obtained, however, that all the cells in each of the clones will support infection when exposed to a relatively high multiplicity of infection and that the clones that were least effective as plaque assay indicators and the least rapid producers of polyhedra produced, on the average, more infectious extracellular particles per cell than did the better indi-

cator lines. These data are inconsistent with the notion that the poor indicator lines are less able to support infection and subsequent virus production than the better indicator lines. Attempts to establish an alternative assay to check the fidelity of this plaque assay are currently being made.

It was found, also, that the extracellular PFU lose considerable activity when frozen at −90 C for just 9 days. Also at least 30% of PFU activity is lost when virus is held at 28 C for over 10 h. This observation is significant in view of the fact that the standard procedure for growing nuclear polyhedrosis virus is to collect the medium of an infected culture after 3 to 6 days at 28 C (2, 4, 6, 7). We have shown that the highest yield of extracellular PFU occurs when 70 to 90% of the cells in the infected culture contain polyhedra, usually 24 to 48 h after infection.

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