The Cellular Substrate: A Very Important Requirement for Baculovirus *in vitro* Replication

Herbert G. Miltenburger, Werner L. Naser, and Jeanne P. Harvey

Institut für Zoologie, Technische Universität Darmstadt, Schnittspahnstr. 3, D-6100 Darmstadt

Jürg Huber and Alois M. Huger

Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für biologische Schädlingsbekämpfung, Heinrichstr. 243, D-6100 Darmstadt

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We established more than 200 primary cell lines of *Cydia pomonella* (codling moth). 81 of them were selected and screened for replication of two baculoviruses (from two different subgroups): the *Choristoneura murinana* NPV and the *Cydia pomonella* GV. Although all these cell lines had been derived from the same insect species, they varied largely in their response to challenge with the NPV. Most of them showed CPE or produced different amounts of polyhedra. Interestingly, we also found a few cell lines that were permissive for GV replication. To our knowledge this is the first time that GV replication in cell lines has been obtained. Our results show that cell line properties are most important for baculovirus *in vitro* replication.

Introduction

Baculoviruses (BV) are of great importance as promising microbial insecticides, as subjects of basic research and as powerful cloning vectors [1].

The virus family Baculoviridae contains two main subgroups: the nuclear polyhedrosis viruses (NPV; subgroup A) and the granulosis viruses (GV; subgroup B) [2]. During the *in vivo* replication of these BVs tissue-dependent responses are well known. Most NPVs replicate in gut cells, tracheal matrix, hemocytes, epidermal tissues and fat body cells, whereas GV replication is sometimes restricted to gut and fat body cells.

Despite the fact that these tissue-specific responses occur in the living insect larvae [3] little attention has been paid to the implications of this for BV *in vitro* replication.

The influence of the cellular substrate on NPV replication *in vitro* has been studied in only a few investigations [4, 5]. As it was shown there, the properties of a given cell line used influence both polyhedral yield and $TCID_{50}$ values.

Although *in vitro* systems for the replication of NPVs have been known for more than ten years, analogous systems have not yet been described for GV *in vitro* replication. There are at least three major possibilities to explain the failure to achieve a GV *in vitro* replication system: 1. The virus used as inoculum is not infectious. 2. The medium used is not appropriate for GV replication, either due to the lack of salts, nutrients, hormones and "factors", or due to "antiviral activity" of one of the components, *e.g.* from fetal calf serum. 3. The cell line tested for GV replication is not able to promote viral reproduction because of missing virus receptors or because of missing enzymes that are required for GV replication.

In order to learn more about the role of cell lines in BV replication we developed a model system centering around *Cydia pomonella* (Cp). This insect species is susceptible to infection by two baculoviruses: by the *Choristoneura murinana* (Cm) NPV (a subgroup A virus) and the *Cydia pomonella* GV (a subgroup B virus). By establishing more than 200 "primary" cell lines (PCLs) from Cp we have been able to study the differences in response to BV challenge that PCLs derived from one insect species can exhibit.

As we demonstrate in this communication the cellular substrate is a vitally important component in BV *in vitro* systems.

Reprint requests to H. G. Miltenburger.

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Material and Methods

1. Tissue culture medium

The Cp-medium formulation is based on that described by Grace [6]. It has been adapted to the requirements of Cp cells by Dr. Peters, Wageningen, The Netherlands (personal communication), and was further modified in our laboratory. As this medium is very rich in salts (see Table I), crystallizing of media components sometimes caused problems. The greatest problems occurred in cultures that were growing slowly and/or had to be passaged by scraping. Therefore the Cp medium was further modified to give the "Cp$^-$" medium, which contained only half the amount of four salts (see * in Table I). Both media were supplemented with 10% fetal calf serum.

2. Establishment of new cell lines

a) From larval cells

Primary cultures were derived from tissues and hemocytes of L4/L5 Cp larvae. A total of about 900 hemocyte and 100 explant primary cultures from various tissues were set up. The animals were surface sterilized with Rinaldini's solution [7] to which 325 mg/l penicillin and 550 mg/l streptomycin had been added. Minced tissue pieces (about 1 mm^3) attached to the bottom of plastic petri dishes within 4–6 h while hemocytes needed up to 24 h for attachment. 1 µl of Cp hemolymph contained about $1-2 \times 10^4$ hemocytes, and about 5×10^4 hemocytes per ml medium were required for survival of sufficient numbers of cells. Primary cultures were also obtained by trypsin treatment (0.6–2.0% trypsin; 30–60 min; RT) from fragments of the following tissues and organs: intestine, dorsal vessel, fat body, gonades, muscle, salivary glands, tracheae, and epidermis.

b) From embryonic cells

Embryonic Cp cells were obtained from Cp eggs. In most cases the eggs were 2–3 days old, but older eggs were also used. About 200 eggs were surface sterilized by incubation in 2% sodium hypochlorite for 10 to 15 min. The eggs were removed from the cellophane sheet on which they had been laid, and transferred into a funnel provided with a mesh (0.01 mm^2) of stainless steel. There they were washed for 3–5 min with 70% ethanol followed by tissue culture medium to remove the ethanol. The egg shells were broken with a pair of forceps and their contents were passed through the steel mesh. The homogenate, containing single cells and small cell aggregates, was suspended in 5 ml Cp-medium and seeded out into a 25 cm^2 tissue culture flask.

Table I. Cp medium (= IZD Lp 03).

	mg/ml		mg/l
KCl	2870.0	L-Proline	175.0
$CaCl_2 \times 2H_2O$*	1320.0	L-Phenylalanine	75.0
$MgCl_2 \times 6H_2O$*	2280.0	DL-Serine	550.0
$MgSO_4 \times 7H_2O$*	2780.0	L-Threonine	87.5
$NaH_2PO_4 \times 2H_2O$*	1140.0	L-Tryptophan	50.0
$NaHCO_3$	350.0	L-Tyrosine	25.0
		L-Valine	50.0
L-Arginine × HCl	350.0		
L-Aspartic acid	175.0	*p*-Aminobenzoic acid	0.010
L-Asparagine	175.0	Biotin	0.005
L-Alanine	112.5	D-Ca-Pantothenate	0.010
β-Alanine	100.0	Choline chloride	0.100
L-Cystine	11.0	Folic acid	0.010
Glutamic acid	300.0	*i*-Inositol	0.010
Glutamine	300.0	Nicotinic acid	0.010
Glycine	320.0	Pyridoxine × HCl	0.010
L-Histidine × HCl	1250.0	Riboflavin	0.010
L-Isoleucine	25.0	Thiamine × HCl	0.010
L-Leucine	37.5		
L-Lysine × HCl	312.5	Glucose	1000.0
L-Methionine	25.0	Tryptose	2600.0

The medium is supplemented with 10% fetal calf serum. The pH value is adjusted to 6.4 with 1 N KOH.
* see Material and Methods.

3. Methods of passaging

a) Shaking (= PS in Table II); unattached or weakly attached cells were suspended by shaking the flasks vigorously and then transferred with a pipet into a new flask.

b) Pipetting (= PP in Table II); cells that remained attached after shaking were suspended by vigorous pipetting and transferred into a new flask.

c) Scraping (= PK in Table II); one group of cells that grew firmly attached to the bottom of tissue culture flasks was scraped off with a rubber police-

Table II. PCL-screening. The first number represents the set-up number, whereas the next numbers followed by two capital letters indicate the levels of the respective modes of passaging (see Material and Methods). A "–" at the end of a PCL designation means that this PCL grows in Cp^--medium. An "○" stands for not tested, whereas the intensity of reactions or properties is indicated by the number of plusses.

Cell lines tested	Morphology	Adherance	Splitting ratio/ weeks	Response to baculoviruses			
				CmNPV LM	CpGV LM	CpGV 2D9	CpGV 24C4
14-34PT	small, round, spindleshaped	++	1:2/1	+	–	–	–
14-10-PT-25PS	small, spindleshaped	–	1:5/1	+	–	–	–
21-1PC-3PS-21PC	long, spindleshaped	++	1:2/1	+	–	–	–
11a-35PC	large, round-spindleshaped	++	1:4/1	–	–	–	–
6-1PC-3PP-23PC	large, epithelial-like	++	1:4/1	+	–	–	–
35-21PC	epithelial-like	++	1:2/1	++	–	–	–
12-25PC	round	++	1:3/1	–	–	–	–
22-20PC$^-$	faint, no clear cell border	++	1:2/1	++	–	–	–
4-1PS-2PT-18PC	fibroblast-like	++	1:3/1	CPE	–	–	–
25a-33PC	epithelial-like	++	1:7/1	+	–	–	–
30a-20PC$^-$	faint, fibroblast- a. epithelial-like	++	1:2/1	+++	–	–	–
24a-26PC	large, epithelial-like	++	1:2/1	+	–	–	–
33b-19PK$^-$	net-like, fibroblast-like	++	1:4/1	+++	++	++	++
32b-8PS-8PK$^-$	small, very long, net-like	++	1:2/2	+++	–	–	–
27-1PC-19PK$^-$	very long, net-like	++	1:2/1	+++	–	–	–
3-1PC-26PS	faint, net-like	+	1:3/1	+	–	–	–
8-PK-31PS$^-$	large, round-spindleshaped	–	1:4/1	–	–	–	–
32-PC-PS-18PK	long, contrasty, fibroblast-like	++	1:2/2	+	–	–	–
8-PS-2PC-27PS$^-$	round-spindleshaped	–	1:4/1	+	–	–	–
25-11PK$^-$	very faint, many fat droplets	++	1:2/3	+++	–	–	–
9-12PK$^-$	mixed population of cells	++	1:2/3	+	–	–	–
14-1PC-9PK$^-$	very long, net-like	++	1:2/4	++	–	–	–
16-2PC-5PK$^-$	very faint, long, net-like	++	1:2/4	++	–	–	–
30-14PK$^-$	faint, fibroblast-like, fat droplets	++	1:2/2	+++	–	±	±
14-1PC-1PS-17PK	faint, net-like	++	1:2/2	++	–	±	–
8-1PS-11PK$^-$	small, fibroblast-like	++	1:2/3	+	–	–	–
10-1PC-14PK$^-$	faint, fibroblast-like, net-like	++	1:2/2	+	–	±	±
11-1PC-14PK$^-$	very long, fibroblast-like	++	1:2/2	+	–	+	+
9a-19PK$^-$	round	++	1:2/2	+	–	–	–
8-1PC-24PP$^-$	small, round	±	1:3/1	–	–	–	–
11-2PC-1PP-24PK$^-$	fibroblast-like, with fatty clumps	++	1:3/1	+	–	–	–
11-1PC-11PS-9PT$^-$	small, spindleshaped	++	1:2/2	+	–	–	–
24a-2PC-18PT	large, round-spindleshaped	++	1:2/2	–	–	–	–
9a-8PT-1PS-6PT	spindleshaped	++	1:2/3	–	–	–	–
30a-18PC$^-$	faint, epithelial-like, net-like	++	1:2/2	++	–	–	–
10-31PC$^-$	fibroblast-like, piling up	++	1:2/1	+	–	–	–
11b-17PC	epithelial-like, some w/fat droplets	++	1:2/1	+	–	–	–
28b-1PP-3PC-16PS	lone, spndleshaped	+	1:3/2	+	–	±	–
28b-1PP-27PC	round-spindleshaped	++	1:2/1	++	–	–	–
no cells in that position in Figure 4							
larval PCL #3-50PS	spindleshaped	–	1:5/1	CPE	–	–	–
larval PCL #2-48PS	round-spindleshaped	–	1:10/1	CPE	–	–	–
larval PCL #1-48PS	large, round	–	1:12/1	CPE	–	–	–
10-13PK$^-$	very long, fibroblast-like	++	1:2/3	+++	–	–	–
10-1PC-42PS	round-spindleshaped	+	1:5/1	CPE	–	–	–
5-9PK-7PS$^-$	small, clumpy	–	1:5/1	+	–	–	–

Table II. (continued)

Cell lines tested	Morphology	Adherance	Splitting ratio/ weeks	Response to baculoviruses			
				CmNPV LM	CpGV LM	CpGV 2D9	CpGV 24C4
6-35PS	small, spindleshaped	−	1:6/1	−	−	−	−
32b-36PS⁻	round	±	1:5/1	−	−	−	−
14c-32PS⁻	round	+	1:4/1	−	−	−	−
6-1PC-8PK-32PS⁻	large, round	−	1:5/1	−	−	−	−
5-1PK-31PS	round-spindlesh., mixed population	±	1:5/1	CPE	−	−	−
9a-30PT	small, round	++	1:5/1	CPE	−	−	−
35-3PC-26PP⁻	small, spindleshaped	±	1:3/1	−	−	−	−
11a-36PK⁻	very small, round	+	1:2/1	CPE	−	−	−
27-2PC-40PS	large, spindleshaped	−	1:10/1	+	−	−	−
11-1PC-6PS-21PK⁻	net-like, fibroblast-like	++	1:2/1	++	+	+	+
6-2PC-33PS	large, round	−	1:4/1	CPE	−	−	−
6-1PC-34PK	large, spindleshaped	+	1:3/1	−	−	−	−
14-1PP-6PS-21PK⁻	long, fibroblast-like, net-like	++	1:2/1	+	−	−	−
14-9PP-16PK⁻	spindlesh., net-like, clumpy		1:2/1	+++	−	+	+
9a-1PS-35PP	round	−	1:12/1	+	−	−	−
11-1PP-40PS	round	−	1:15/1	−	−	−	−
27-1PC-37PS	round-spindleshaped, small	+	1:4/1	+	−	−	−
23-22PS	faint, epithelial-like	+	1:4/1	CPE	−	±	−
24-PC-37PS⁻	round	−	1:4/1	+	−	−	−
7-9PK-7PS	round	−	1:5/1	−	−	−	−
24-39PK	small, round	+	1:6/1	cells died before tested			
11-4PC-28PS	spindleshaped, clumpy	−	1:5/1	+	−	−	−
15-1PC-41PP⁻	small, round	±	1:5/1	−	−	−	−
11a-1PC-43PS	large, round	−	1:7/1	+	−	−	−
11a-2PC-15PK-17PS	round	±	1:4/1	−	−	−	−
11-1PK-2PT-35PS	round-spindleshaped	−	1:6/1	−	−	−	−
25-1PC-40PS⁻	round-spindleshaped	±	1:5/1	++	−	−	−
16-16PK⁻	faint, very long, fibroblast-like	++	1:2/2	CPE	−	−	−
11b-2PC-27PS	round-spindleshaped	−	1:5/1	−	−	−	−
larval PCL #4-46PS	large, spindleshaped	−	1:5/1	−	−	−	−
34-1PC-12PS-2PK-23PS	small, clumpy	−	1:12/1	−	−	−	−
3-1PC-37PS	small, round, mixed population	±	1:12/1	−	−	−	−
21-1PC-8PS-17PK⁻	faint, net-like	++	1:2/1	+	−	−	−
16-4PC-28PS⁻	small, round	±	1:3/1	−	−	−	−
32-1PC-42PS⁻	large, round-spindleshaped	±	1:6/1	++	−	−	−
15-1PC-40PS	small, spindleshaped	±	1:9/1	−	−	−	−
Cp 169 (Hink)	standard sized spindles	−	1:5/0.5	+	−	○	○
IZD-Cp2202	similar to Cp169	−	1:7/0.5	+	−	○	○
IZD-Cp0508	half as big as Cp169	−	1:5/0.5	++	−	○	○
IZD-Cp1508	mixed pop. round-spindleshaped	−	1:3/0.5	CPE	−	○	○

man. The resulting cell clumps were dissociated by pipetting prior to transfer.

d) Trypsin treatment (= PT in Table II); another group of firmly attached cells was incubated with 1.5 ml trypsin (0.2% in Ca−Mg-free salt solution) for 15 min, then the enzyme was inactivated by the addition of 3.5 ml Cp-medium (Table I). The cells were finally suspended by pipetting, pelleted, resuspended in Cp medium, and transferred into the next tissue culture flask.

e) Collagenase treatment (= PC in Table II); a final group of firmly attached cells was detached by incubation with collagenase (0.2%, 60 min; 27 °C). These cells were washed once in medium to remove the collagenase and seeded out into new flasks together with about 2 ml of conditioned medium taken from the same PCL.

3. Screening of the PCLs for BV replication

a) Preparation of the cell lines

81 PCLs were seeded out into 4 wells each of a 24 well tissue culture plate. An attempt was made to have all the PCLs half-confluent and in the logarithmic phase at the time of inoculation.

b) Inoculation

Previous experiments showed that some of the PCLs supported CmNPV replication (data not shown) and yielded infectious supernatant that was used here as inoculum. 100 µl of tissue culture supernatant (CmNPV) were added to the wells containing 1 ml of medium. The PCLs that showed the best results in the first screening experiments were further tested for their stability with respect to production of high amounts of normal-sized polyhedra. This was done by passaging CmNPV (1:5 or 1:10) for at least five subsequent times through each of these PCLs.

Preparation of the CpGV inoculum (derived from infectious hemolymph) is described elsewhere [8]. In order to improve the infection the medium was first removed from the wells. The cells were treated with 100 µl of inoculum for 4 h at 27 °C. Finally 1 ml of medium was added and the cells were then examined in two days intervals.

4. Immuno-dot-assay

a) Monoclonal antibodies

Monoclonal antibodies were prepared according to standard procedures [9] against CpGV released and purified from capsules. The monoclonals used in the assays described here had been selected by indirect ELISA for reacting against a CpGV structural protein and not reacting against granulin. In immunofluorescence studies on thin sections of healthy and infected larvae, their specificity for CpGV had been further proven (data not shown).

b) Assay procedure

The cells of the PCLs tested were harvested 7–8 days after inoculation, washed once with phosphate buffered saline (PBS) pH 7.4, resuspended in 50 µl PBS and spotted onto nitrocellulose filters. For each cell line 3 replicates of 5 µl were applied. A 15 min incubation in 3% casein in PBS was used to block the remaining protein binding sites on the nitrocellulose. The three filters were rinsed with PBS and incubated in 20 ml of 1:200 diluted ascites fluid of two positive (2D9 and 24C4) and one negative monoclonal antibody (1C9). After 1 h at room temperature the filters were washed three times (PBS), incubated in rabbit-antimouse-horseradish peroxidase-conjugate (60 min; RT), washed 5 times and finally incubated for 30 min in substrate solution (25 µg/ml *o*-dianisidine and 0.01% H_2O_2 in PBS).

5. Isoenzyme analysis

Isoelectric focusing was performed in thin layer polyacrylamide gels (LKB Instrument GmbH, D-8032 Gräfelfing, FRG). Staining for esterase isoenzymes was done according to the method of Simms [10] as described earlier [11].

Results

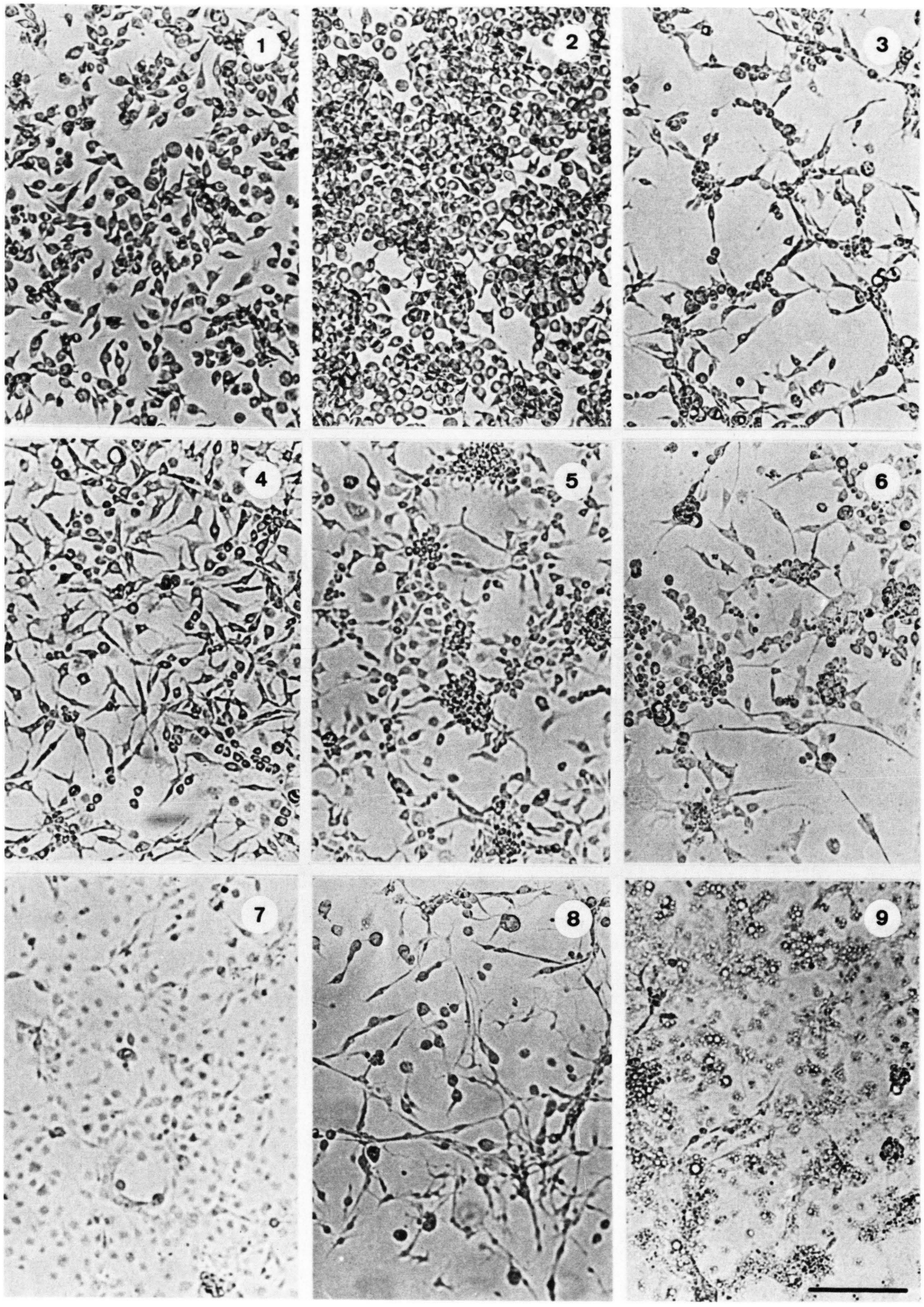
The primary cell lines obtained

Most of the hemocyte cultures and all tissue explants degenerated within days or weeks. However, one hemocyte primary culture survived and gave rise to four primary cell lines (*cf.* larval PCLs in Table II). The three continuous cell lines IZD-Cp 2202, IZD-Cp 0508, and IZD-Cp 1508 were also established from hemolymph cells in 1973 and 1974 (unpublished).

All the other PCLs were set up from embryonic Cp cells. In all of these primary cultures dividing cells and growing colonies were observed, and in 32 out of 35 set-ups healthy looking cells grew to a density that allowed their passage. Several different primary cell lines were easily obtained from one primary culture by applying the different subculturing methods. For example in Table II there are 13 PCLs derived from set up no. 11. All of them differ in some of the properties listed there.

Isoenzyme analysis

As can be seen from Table II there was a wide variation, for all the properties listed, between the different PCLs. However, the PCLs that were passaged by the same passaging procedure shared more similarities with each other than with PCLs passaged by another procedure. For example, the PCLs passaged by scraping usually grew relatively slowly and firmly attached to the culture vessel, they usually were faint and fibroblast-to-epithelial-like, and some of them seem to be more appropriate for BV *in vitro* replication (*cf.* Table II). In order to see if some of the "most promising" PCLs might be identical, seven PCLs passaged by scraping and two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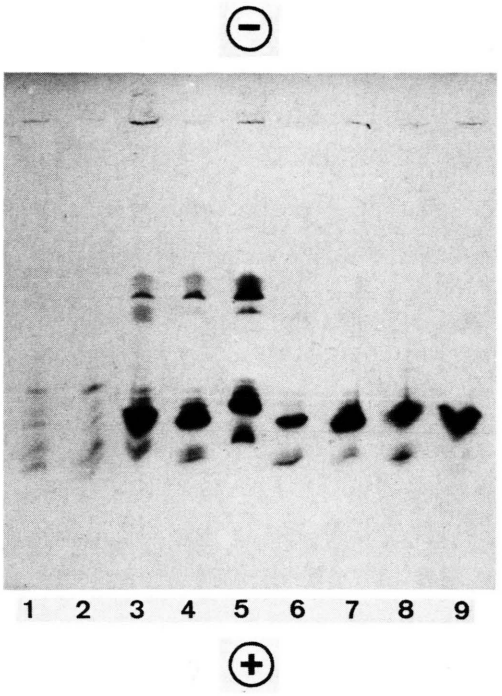

Fig. 2. Esterase isoenzyme patterns. The analyzed cell lines are the same as in Fig. 1.

permanent cell lines have been further characterized. As can be seen from Figs. 1 and 2 there was no similarity between the PCLs and the permanent cell lines with respect to both morphology and the esterase isoenzymes.

The seven PCLs investigated seem to fall into two isoenzyme subgroups formed by 33b-26PK$^-$, 11-1PC-6PS-26PK$^-$ and 14-9PP-25PK$^-$, and by 11-1PC-17PK$^-$, 30-20PK$^-$, 25-14PK$^-$, and 10-14PK$^-$, respectively. Nevertheless it can be seen from Fig. 2 that none of the PCL isoenzyme patterns shown are identical. Therefore, it is very likely, as well, that the PCLs investigated are not identical cell lines.

Response to challenge with CmNPV

Response to infectious hemolymph

The 81 PCls tested for CmNPV replication showed a wide scale of responses to that BV. 23 of them did not seem to be affected at all, while 11 showed cytopathic effects but no remarkable formation of polyhedral occlusion bodies. In about half of the PCLs polyhedra were produced, but in only 8 of them this polyhedra formation was judged to be very good (three plusses in Table II); that means most cells were infected and contained a high number of small polyhedra (*cf.* Figs. 3 a, b).

Fig. 1. Morphological comparison of two continuous and seven primary cell lines. The cell lines, all shown at the same magnification, are: 1 = IZD-Cp 0508; 2 = Cp 169 (Hink); 3 = IZD-Cp 33b-26PK$^-$; 4 = 11-1PC-6PS-26PK$^-$; 5 = 14-9PP-26PK$^-$; 6 = 11-1PC-16PK$^-$; 7 = 30-20PK$^-$; 8 = 10-14PK$^-$ and 9 = 25-14PK$^-$. Bar represents 50 µm.

Stability of CmNPV replication

Those PCLs that produced many regular-sized polyhedra in the first screening assay (+++ in Table II) and one additional PCL (15-2PC-6PK$^-$) were subsequently tested for their stability in polyhedra production.

As can be seen from Table III the 9 PCLs tested responded differently when CmNPV was serially passaged in them. Only three out of 9 proved to be relatively stable for CmNPV replication even after 5 serial passages. Two other PCLs looked very promising also, but as they grew rather slowly, they were tested for only three serial CmNPV passages. It is also obvious from Table III that the decrease in CmNPV polyhedra production in the four unstable PCLs occurs rather rapidly, as it is visible already after the third *in vitro* passage.

Response to challenge with CpGV

Light microscopical observations

In one of the PCLs tested (33b-19PK$^-$) changes were obvious. About half the cells showed CPE, *i.e.* the usually long fibroblast-like cells rounded up. The typically infected cells were round, of brownish

Table III. Stability of CmNPV production in 9 PCLs.

Name of the PCL tested	CmNPV polyhedra produced after infection with		
	Infect. hemolymph	2nd pass. TCSV	4th pass. TCSV
10-11PK$^-$	+++	+++	not yet tested
14-9PP-25PK$^-$	+++	++	+
15-2PC-6PK$^-$	+++	+++	not yet tested
25-15PK$^-$	+++	+++	+++
27-1PC-19PK$^-$	+++	++	+
30-20PK$^-$	+++	+++	+++
30a-20PC	+++	+	+
32b-8PS-PK$^-$	+++	+	+
33b-20PK$^-$	+++	+++	+++

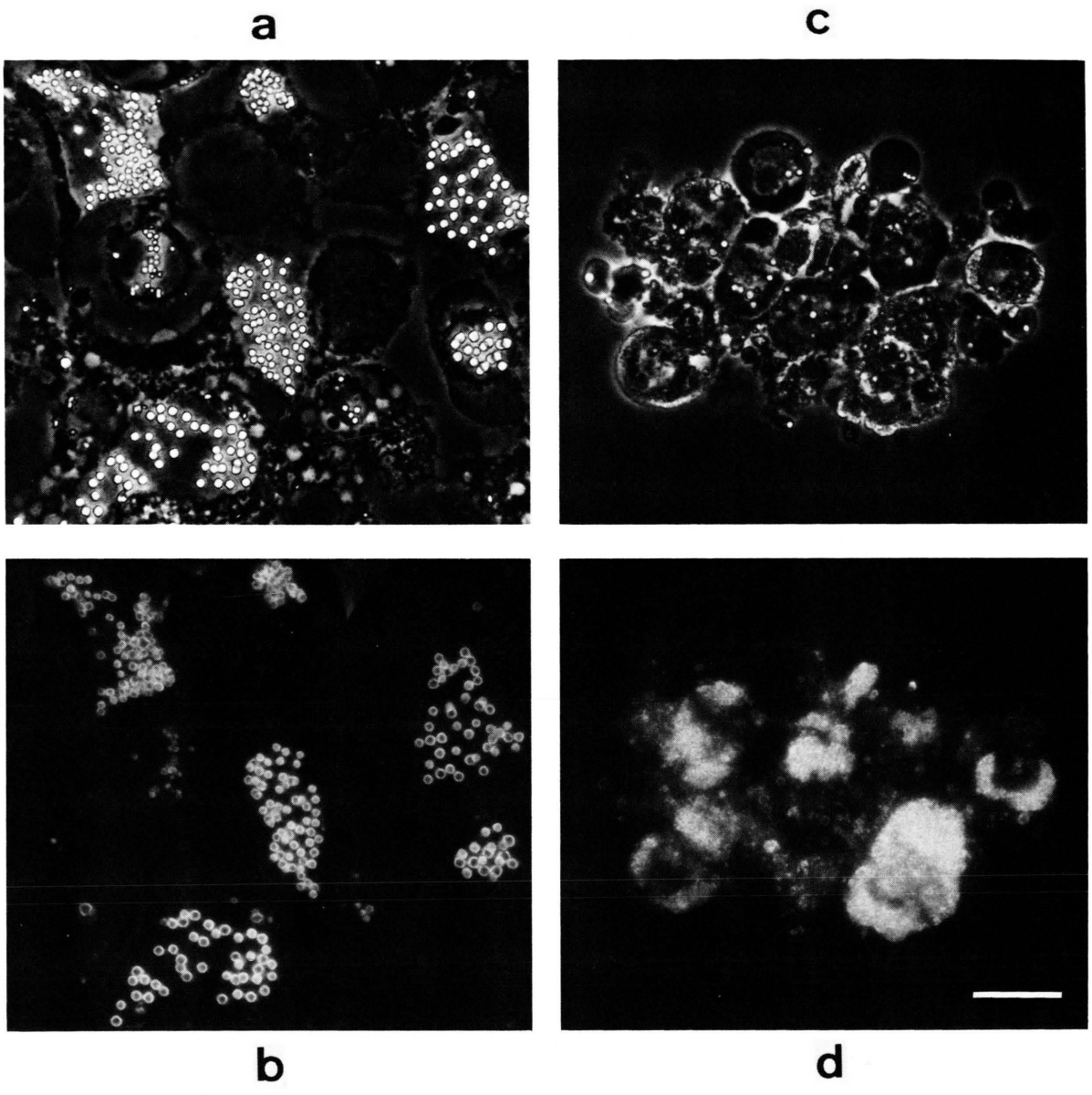

Fig. 3. IZD-Cp 33b cells infected with CmNPV and CpGV. Cells of IZD-Cp 33b, at PK-level 28, were infected with CmNPV (a + b) or CpGV (c + d) and photographed when occlusion body formation was obvious. The same microscopical fields are shown in a+b and c+d, respectively (a+c phase contrast, b+d dark field illumination). The bright cell contents in dark field illumination are the polyhedral occlusion bodies (b) or massive accumulations of capsules (d). Bar represents 20 µm.

color and small particles inside showed rapid Brownian movement. Electron microscopy showed that the small particles were virus-containing capsules of the size and shape typical for CpGV [8].

In a second PCL (11-1PK-6PS-21PK$^-$) cytopathic effects were also visible, but only a few of the cells were brown and showed the rapid Brownian movement. In all the other PCLs 8 days after inoculation there was no cytopathic change detectable.

CpGV-detection by monoclonal antibodies

All the PCLs included in the screening experiment were also tested for the production of CpGV-

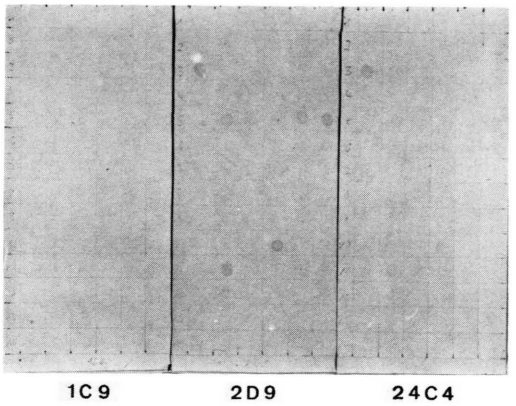

Fig. 4. Immuno-dot-assay. Three replicates of each PCL sample were spotted onto nitrocellulose filters and analyzed with the monoclonal antibodies 1C9, 2D9 and 24C4, respectively. Each of the 14 horizontal lines contains six samples. The sequence of the samples from position 1 to 82 (*i.e.* dot four in line 14) is the same as in Table II.

specific proteins by two positive monoclonal antibodies 2D9 and 24C4 and a negative monoclonal antibody 1C9 as described in Material and Methods. The results obtained are shown in Fig. 4. They are consistent with the light microscopical observations with respect to the two PCLs that showed CPE and typically infected cells. Both of the two positive monoclonal antibodies reacted also with samples of the two PCLs 11-1PC-14PK$^-$ and 14-9PP-16PK$^-$, and weakly (± in Table II) with samples of 10-1PC-14PK$^-$ and 30-14PK$^-$. However, only 2D9 reacted at a low level (± in Table II) with samples of three other tested PCLs: 14-1PC-1PS-17PK$^-$, 28b-1PP-3PC-16PK$^-$, and 23-22PS.

Discussion

NPV *in vitro* replication systems have proven to be very important and valuable tools in NPV research. Such *in vitro* systems consist mainly of three components: the viral inoculum, the medium, and the cellular substrate. For the second largest subgroup of the virus family Baculoviridae, the GVs, no *in vitro* systems had been obtained so far. As in the past, not very much attention had been paid to the roles that cell lines might play in NVP *in vitro* replication, and as the lack of some cellular properties might have been responsible for failures in obtaining GV *in vitro* replication, we developed a new model system, to learn more about the importance of the cellular substrate for NPV and GV replication. Since the larvae of *Cydia pomonella* (codling moth) are infected by a NPV and a GV, it seemed promising to establish many new PCLs derived from this insect species.

It was rather difficult to establish PCLs of larval origin and in only one set-up out of about one thousand explants healthy growing cells were obtained, which ultimately gave rise to 4 different PCLs. On the other hand, it was unexpectedly easy to establish PCLs from Cp eggs according to the method described. Also, it proved to be not very difficult to obtain several different PCLs from one initial primary culture. From about 200 PCLs over 90 were tested for CmNPV and CpGV-replication, 81 of which are included in Table II. It is possible that some of the PCLs are identical, but probably not very many are. As can be seen from the data given on morphology, adherance and growth rate in Table II, most of the PCLs derived from one set-up show significant differences. On the other hand, the PCLs passaged by the same techniques, especially those passaged by scraping with a rubber policeman, are somewhat similar. We therefore conducted an esterase isoenzyme analysis on seven out of those PCLs that looked promising either for CmNPV or CpGV replication. As can be seen from Fig. 2 the PCLs investigated fall into two isoenzyme subgroups, but there are no identical isoenzyme patterns. Therefore it is very unlikely that many of the PCLs investigated are identical.

When we challenged 81 Cp PCLs, *i.e.* PCLs derived from the same insect species, with CmNPV we found a wide variation of responses. Whereas some PCLs were not affected at all, others reacted with CPE, low and intermediate levels of polyhedra production, and again others produced many polyhedra with a high percentage of cells being infected. As tissue dependent interactions are well-known in the insect larva, this might be an indication that some of the PCLs share some properties with differentiated larval tissues.

From previous experiments (unpublished data) with CmNPV in the permanent cell line IZD-Cp 0508 it was known that this BV is a rather slow replicating virus, with CPE and polyhedra formation occurring relatively late compared to the AcNPV-IZD-Mb 0503 system (36 and 72 h compared to 12 and 24 h, respectively) [12]. Perhaps this slow viral replication contributed to the rapid appearance of

abnormal infections (*i.e.* only a few and very large polyhedra per cell) after serial passage of CmNPV. This phenomenon greatly resembles the well-known generation of the so-called FP variants that have been described [*e.g.* 13, 14]. In order to see if cellular properties might also contribute to that phenomenon, those 8 PCLs that seemed to be very good for CmNPV replication and polyhedra formation (*cf.* Table III) together with another promising PCL, 15-2PC-6PK$^-$, were tested for their stability in CmNPV polyhedra production.

We found that even those PCLs initially showing a very good response to challenge with CmNPV behaved differently when CmNPV was serially passaged 5 times in the same PCL. The CmNPV polyhedra production was rather stable in at least three (possibly five) out of 9 PCLs, whereas it proved to be rather unstable in 4 PCLs. This might mean that cellular properties could also be important for the formation of FP variants of BVs during *in vitro* passages.

Properties of the cellular substrate also seem to be the most important requirements for working GV *in vitro* replication systems.

Only two out of 81 PCLs showed changes after inoculation with CpGV detectable with the light microscope. It has been shown in the electron microscope that these infected cells contain many regular shaped and sized capsules [8]. Consistent with the light microscopical observations it was shown by monoclonal antibodies that CpGV specific proteins are produced in these two PCLs (*cf.* Table II and Fig. 4).

The clearly positive reactions (+ in Table II) with monoclonal antibodies against CpGV-infected samples of 11-1PC-14PK$^-$ and 14-9PP-14PK$^-$ would also indicate that at least two more PCLs produce some CpGV. However, as we concentrated on the two most promising PCLs in the meantime, this question has not yet been answered. Nevertheless the data shown imply that the cellular substrate is of great importance for BV *in vitro* replication in various respects and deserves more attention.

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