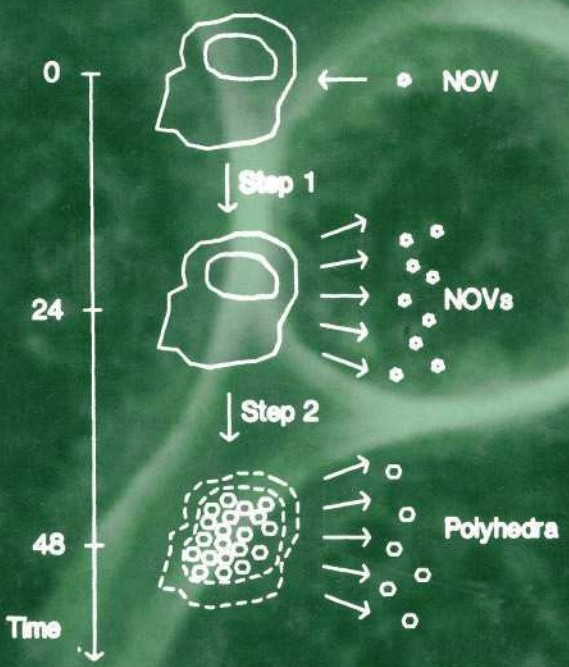
Current Applications of Cell Culture Engineering – Vol. 2

Insect Cell Cultures

Fundamental and applied aspects



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INSECT CELL CULTURES: FUNDAMENTAL AND APPLIED ASPECTS

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Insect Cell Cultures: Fundamental and Applied Aspects

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Special Issue

Preface

The remarkable growth and application of insect cell culture research during the past decade has been driven by several biotechnological advances. Of greatest significance was the development in the 1980's of the application of recombinant DNA technology to express cloned genes in insect host cells. The emergence of the baculovirus-insect cell system as a versatile gene expression tool resulted from intensive and elegant studies on the molecular biology of baculoviruses, and the establishment of new insect cell culture systems which supported high levels of protein expression. The baculovirus expression system has not only become an important tool for research, but is now accepted as an important technology for the commercialization of products for use in agriculture and human health.

Insect cell cultures fundamental and applied aspects provides a comprehensive review of the major advances in insect cell culture research. The book is divided into five sections which systematically approach the topic, from the basic principles of cell culture technology through the concomitant economic and regulatory issues that are important aspects of the commercialization of bio-based products. All chapters in this volume are written by the foremost authorities in the field who have contributed original research in insect cell and molecular biology, chemical engineering, and baculovirology. The first part is devoted to an in-depth review of basic insect cell culture and cell biology with chapters on new advances in transgenic cells and novel media formulations. Part two presents an exceptionally lucid survey of the molecular biology of baculoviruses and their development as expression vectors. These introductory virus chapters are followed by specialized topics on the expression of recombinant proteins and their posttranslational modification. Part three, devoted to review chapters on the application of bioengineering principles for the scale-up of insect cell culture, also discusses the differences between invertebrate and vertebrate cell culture and the impact of cell bioreactor design, shear force concerns, nutrient and oxygen requirements that are specific for insect cells. Parts four and five detail examples of specific commercial products produced in insect cell systems. The book concludes with an exhaustive consideration of the economic and regulatory issues that have to be considered by industry and government agencies. The topics discussed are interrelated through a focus on the insect cell culture-baculovirus system and the prominent role it plays in basic biological research and biotechnology.

The book benefits from clear and precise writing throughout. All chapters are well documented with figures, illustrations, and comprehensive bibliographies. This authoritative volume will serve as a standard information resource on insect cell culture. The book will become the benchmark reference manual for students and researchers in academia and government laboratories requiring information on how insect cell systems are being applied in basic research and biotechnology applications. In particular this treatise will be an invaluable source of knowledge to industrial scientists who are applying the baculovirus-cell culture systems for commercial purposes.

Robert R. Granados
Ithaca, New York
March, 1996

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PART I

INSECT CELL LINES

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Development and characterization of insect cell lines

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Key words: cell line establishment; cryopreservation; insect cells, development of; insect cells, characterization of; isoenzymes; lepidopteran cell cultures

Abbreviations: ICD – Isocitrate dehydrogenase; ME – malic enzyme; PGI – phosphoglucose isomerase; PGM – phosphoglucose mutase.

Introduction

Continuous insect cell lines were first established in culture over three decades ago when Grace (1962) succeeded in growing cells from the *Antherea eucalypti* female moth ovaries. This breakthrough was the result of patience, the availability of antibiotics, and an improved medium. Since Grace's first report on four cell lines, over 400 lines have been established from more that 100 insect species representing every economically important insect order (see Hink, 1972, 1976, 1980; Hink & Bezanson, 1985; and Hink & Hall, 1989 for information on most of these cell lines.) These cell lines have been used in diverse fields of research as described in the other chapters of this book.

In this chapter, I will provide a brief overview of how new cell lines can be established and, once obtained, how they should be handled and characterized. The use of insect cells in baculovirus expression vectors (described elsewhere in this book) has proven to be a blessing to the whole field of insect cell culture by creating a reliable market for insect cell culture media. This means that, where twenty years ago only a couple of companies were selling insect culture media, today every major media company and many smaller companies supply these important components of successful cell culturing.

Since the baculovirus expression vector system has driven the field in recent years, I will be focusing on lepidopteran cells in this chapter. The reader should realize, however, that the techniques that I will describe here are generally relevant to the culture of cells from any insect order.

Development of cell lines

Two factors make primary tissue culture of insects particularly arduous. The first is their generally small size. Grace (1962) overcame this problem by selecting a relatively large moth, but we all cannot be as lucky since our interest may lie with small insect species. The other problem is that insects often live in a dirty environment. Having an insect colony may alleviate both of these problems to some extent. With a colony, a larger number of insects can make up for the relatively small size of the individual. Also, a colony can be cared for in a way to minimize microbial contaminants. I also overcome these problems by setting up primary cultures in small volumes and through the use of antibiotics. While it is generally **not** a good idea to use antibiotics in continuous cell lines (for reasons I shall describe later), they are beneficial in initiating new cell lines. In any case, cell lines have been successfully established from *Trichogramma* wasps (Lynn & Hung, 1991), a genus in which the adult's body is much smaller than the period at the end of this sentence, and from house flies (Eide, 1975) which breed in all kinds of filth.

Selection of medium

The single most important point to consider in attempting to develop a new cell line is the medium. While perhaps the easiest way to do this is with a shotgun approach in which every commercially available medium is tried, a certain amount of thought can go into selecting the order in which these are tried. Many commercial media are sold specifically for Lepidoptera. These range from the "old standby" of Grace's medium (sold by most major media manufacturers) to highly defined, serum-free media such as ExCell 401 (JRH Biosciences, Lenexa, KS[1]), SF–900 (GIBCO, Grand Island, NY) and Insect-Xpress (Whittaker, Walkersville, MD). I personally prefer a modified formulation of BML/TC–10 (Gardiner & Stockdale, 1975) sold commercially as TC–100 (GIBCO, JRH, Sigma Chemical Co., St. Louis, MO and others) to which I add additional peptides (such as 1.25% phytone peptone (BBL Microbiological Associates, Gaithersburg, MD) and 0.075% liver digest (Oxoid USA, Columbia, MD) or 1.25% peptone #P0521 and 0.075% peptone P7750 (Sigma)) and 5–10% fetal bovine serum (Sigma and many other commercial media companies). The other commonly available media are for dipteran cell lines, such as Schneider's *Drosophila* medium (GIBCO, Sigma, and others) and Shields and Sang's M3 medium for mosquito cell cultures (Sigma).

The main points you should consider in selecting a medium for insects other than these two orders are the pH, osmolarity, and the amount and ratio of the inorganic salts. Although it is somewhat outdated, a useful reference for this purpose is Altman (1961). This paper gives information such as concentrations of inorganic salts, freezing point depression (i.e. osmolality), amino acid concentrations and pH of hemolymph from many insects. Based on the information in Altman's paper, you can compare the values of these factors with published formulations of media to select the most appropriate medium for your insect (or a related species) and make modifications as necessary.

Initiation of primary cultures

I have found the most useful source of cells for developing new cell lines to be embryos, especially if you have a colony of insects available. These can usually be obtained in large quantities and the insect chorion is sufficiently impervious to simple disinfectants (such as 70% ethanol) so these can be used to decontaminate the eggs. The general procedure I use for isolating cells is shown in Figure 1.1 normally submerge insect eggs for 5 to 10 min followed by two rinses in sterile distilled H_2O. You can, at this point, simply disrupt the eggs in culture medium in a tissue homogenizer (Bellco Glass, Vineland, NJ), transfer the cell suspension to a tissue culture petri dish or flask (Corning, Costar, Falcon, and Nunc are common brands of tissue cultureware) and wait for cell attachment. Various other methods have been used to obtain embryonic cells, including dechorionating the eggs with chlorox prior to disrupting or using enzymatic treatments (trypsin, collagenase, hyaluronidase, and elastase have all been used) rather than mechanical disruption.

I obtain the best results by using micro dissecting forceps (Roboz Surgical Instruments Co., Inc., Washington, DC) to mechanically break open the chorion in culture medium after disinfection. The embryos are then teased away from the yolk material and transferred to a standing drop (0.1–0.2 ml) of medium (supplemented with 50 μg gentamicin sulfate/ml) in a 35 mm tissue culture petri dish (Falcon #3001). A microscalpel (Roboz) is used to cut each embryo into 4-8 pieces. During the cutting, many of the tissue fragments become attached to the scratches formed by the scalpel in the plastic, from which they will migrate during subsequent days. I generally use 10–20 embryos for each culture. After cutting up the embryos, the dish is sealed by stretching a 5×75 mm piece of Parafilm® around the edge. The dish is then placed in a tightly sealed plastic container with a small beaker of distilled water, and the entire plastic container is incubated at 27 °C.[2] After 1–2 days, an additional 1.0 ml culture medium is added to the dish. It is resealed with Parafilm and replaced in the plastic container in the incubator.

Patience becomes the greatest virtue at this stage. After an initial period in which the cells migrate from the tissue fragments, little growth may be seen for weeks. During this period, additional culture medium should be added to the dish (about 0.5 ml per week). When the petri dish contains about 3 ml medium, all except 0.5 ml should be replaced with 0.5 ml fresh medium. Prior to making this exchange, the culture

[1] Mention of proprietary or brand names is necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

[2] 27 °C is near optimum for many insects. Your own specific insect may warrant a higher or lower temperature.

Primary Culture Procedure

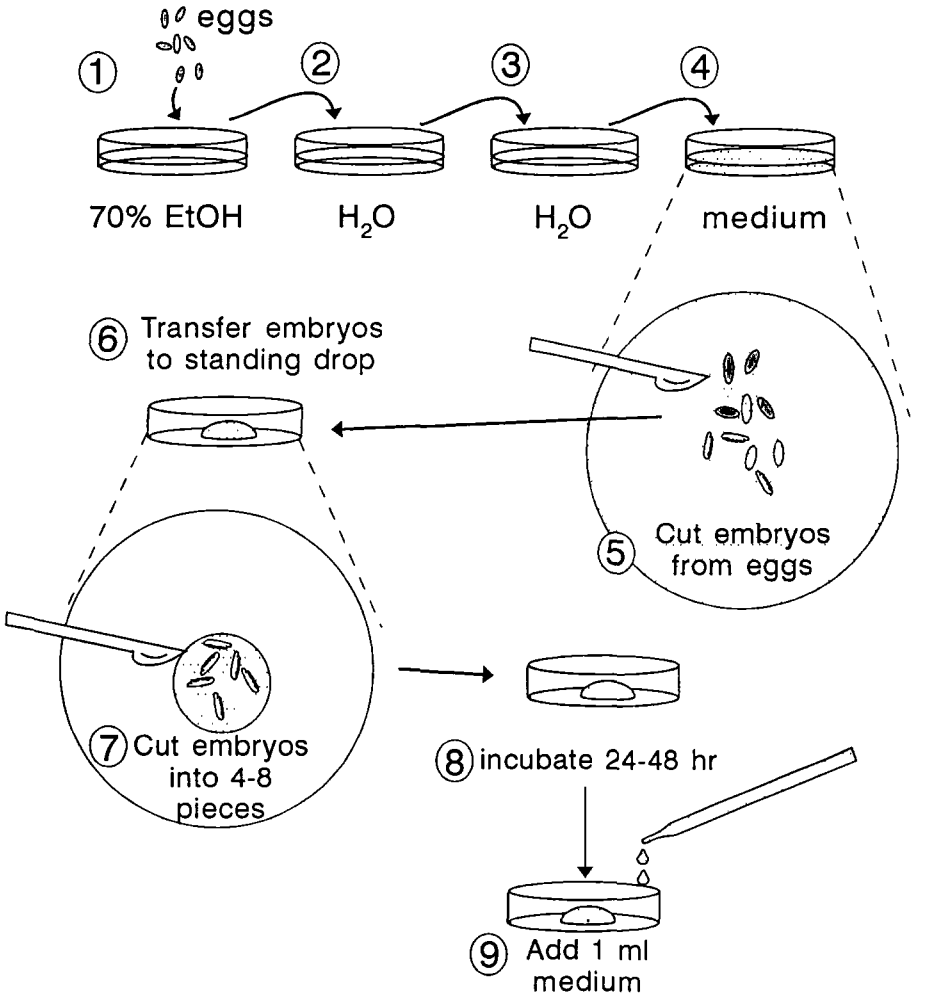

Figure 1. Steps for preparing primary insect cell cultures. Details are described in the text, but the steps include: 1. Disinfect eggs (or whole adult or immature insect if other tissue is desired) in 70% ethanol for 5–10 min. 2. Rinse in sterile distilled water, 5 min. 3. Transfer to fresh sterile distilled water. Hold excess material at this point while proceeding to step 4 with some of the material. 4. Transfer to tissue culture medium containing gentamicin (50 $\mu g\ ml^{-1}$). 5. Place a microscalpel and fine-pointed forceps in 70% ethanol. Ignite alcohol in a flame (Use a small bunsen burner or alcohol lamp. Do not hold instrument in flame, simply ignite and hold at an angle to allow alcohol to burn off. Take care not to hold hand over burning alcohol or to allow alcohol to flow onto your hand.) Use the cooled forceps and scalpel to remove embryos from eggs. 6. Transfer embryos to 35 mm tissue culture petri dish containing a standing drop (0.1–0.2 ml) medium with gentamicin. 7. Cut embryos into 4–8 pieces with microscalpel. 8. Seal petri dish lid to bottom by stretching parafilm® around the edge of dish and incubate at 27 °C in a humidified chamber made from a tightly sealed container (such as Tupperwear®) holding a small beaker of water. 9. 24–48 hr, remove parafilm and add 1.0 ml additional medium containing gentamicin. Reseal with a fresh piece of parafilm and return to humidified container in incubator. (Reprinted with permission from Hackett and Lynn, 1995).

should be examined with an inverted phase contrast microscope. If there are many non-attached cells, the old medium should be transferred to a sterile centrifuge tube. The unattached cells can be recovered by low speed (50 xg, 5–10 min) centrifugation, and then resuspended in the fresh medium before adding it to the original culture. Alternatively, if the original culture contains a substantial number of attached cells, the medium and non-attached cells removed from the primary culture can be transferred to a new dish. I have

often found that these secondary cultures will initiate consistent growth earlier than the primary culture.

This process of adding and replacing medium should be continued as long as living cells are observed in the culture(s). As mentioned above, it may take weeks before the culture contains a substantial number of cells. When the culture reaches about 80% confluence, a subculture may be attempted. The method of subcultivation depends largely on how tightly attached the cells are to the culture dish. I usually attempt a gentle flushing procedure for performing the first subculture. For this, the medium is drawn into a transfer pipet and sprayed across the cell surface to dislodge the cells. The cell suspension is transferred to a new dish (or if there are many cells, to a small (12.5 or 25 cm^2) tissue culture flask with fresh medium. Fresh medium is also returned to the original dish since all the cells are seldom removed by this method.

If few cells are removed by flushing, a more vigorous subculture method can be used. My next attempt normally is to cool the culture at 4 °C for 20 min before using the flushing technique described above. Cooling causes depolymerization of microtubules which are important in attachment of some cells. If cooling does not work, an enzymatic treatment can be used. I first attempt to use collagenase (Worthington Biochemicals, 0.05–0.1 mg ml^{-1} Calcium/Magnesium-free phosphate buffered saline osmotically adjusted to the same concentration as the medium, for lepidopteran cells this is 320–370 mOsm/kg). If collagenase does not remove the cells, I try VMF trypsin (Worthington, 0.05–0.1 mg ml^{-1} saline as described for collagenase). Finally, if all these methods fail to dislodge a substantial proportion of the cells, you can use a cell scraper to remove the cells (a sterile rubber policeman or a specially designed cell scraper available from tissue culture equipment manufacturers). After each of these treatments (flushing, cooling, enzyme) you should wait at least a day before attempting the next harsher treatment since, even if you do not dislodge many cells, you probably cause some cell damage and need to give the culture a chance to recover. You also may find that using these different subculture protocols will result in strains of the original culture with distinct characteristics. (Figure 2).

The secondary cultures are generally treated like the primary culture with fresh medium being added or replaced and subculturing attempted when warranted by cell densities. Eventually with sufficient diligence, you will be able to put the culture(s) on a regular subculture routine. I often find that a cell line will continue to improve in growth rates during the first year or two of regular subculturing. During this period, you are selecting for cells which grow faster, survive the subculture procedure better or, most likely, a combination of these factors. If you have a particular goal in mind of what you want these cells to do for you (virus replication, specific biochemical products, responsivity to hormones, etc.), you should test for the desired properties as soon as you can spare some cells. If you find a culture with the desired characteristics, you should: 1) freeze some cells in liquid nitrogen (see procedure later in this chapter) and 2) attempt to isolate a uniform culture by cloning (Lynn, 1989) or other selection technique. (For example, if you notice cells subcultured by one technique has a greater proportion of desirable cells, use this subculture method to maintain a selection pressure on the cells.)

Maintenance of cell lines

A number of important rules should be followed in maintaining a cell culture laboratory. First, you should **always** use a different bottle of culture medium for each cell line you maintain in the lab. A scandal of sorts exists in cell culture history in which many cell lines (many reported to be normal human diploid) used for experiments were later found to be HeLa cells (cervical cancer cells; Nelson-Rees *et al.*, 1981). The accepted explanation for these mixups was that HeLa cells were maintained in the laboratory where the research was being done and, during subculturing, the bottle of medium shared between the various cultures in the lab was inadvertently contaminated with the HeLa cell line. Since HeLa cells are very vigorous, fast-growing cells, they often outgrew the other cells being kept in the laboratory until they were the only cells present. A similar event occurred in the early history of insect cell culture when Grace (1966) developed an *Aedes aegypti* cell line which subsequently was determined to be *A. eucalypti* cells.

Also, you should only handle **one** cell line at a time. I maintain from 10–20 different cell lines in my lab at any one time. It is obviously useful to handle these cultures at the same time for use in initiating experiments, but, as Einstein reportedly said, time is relative. When handling your primary stock of cells (as opposed to cells being used in "deadend" experiments), you should only have that cell line and its own bottle of maintenance medium (use a different bottle of medium for experiments) in the transfer hood at the time. This

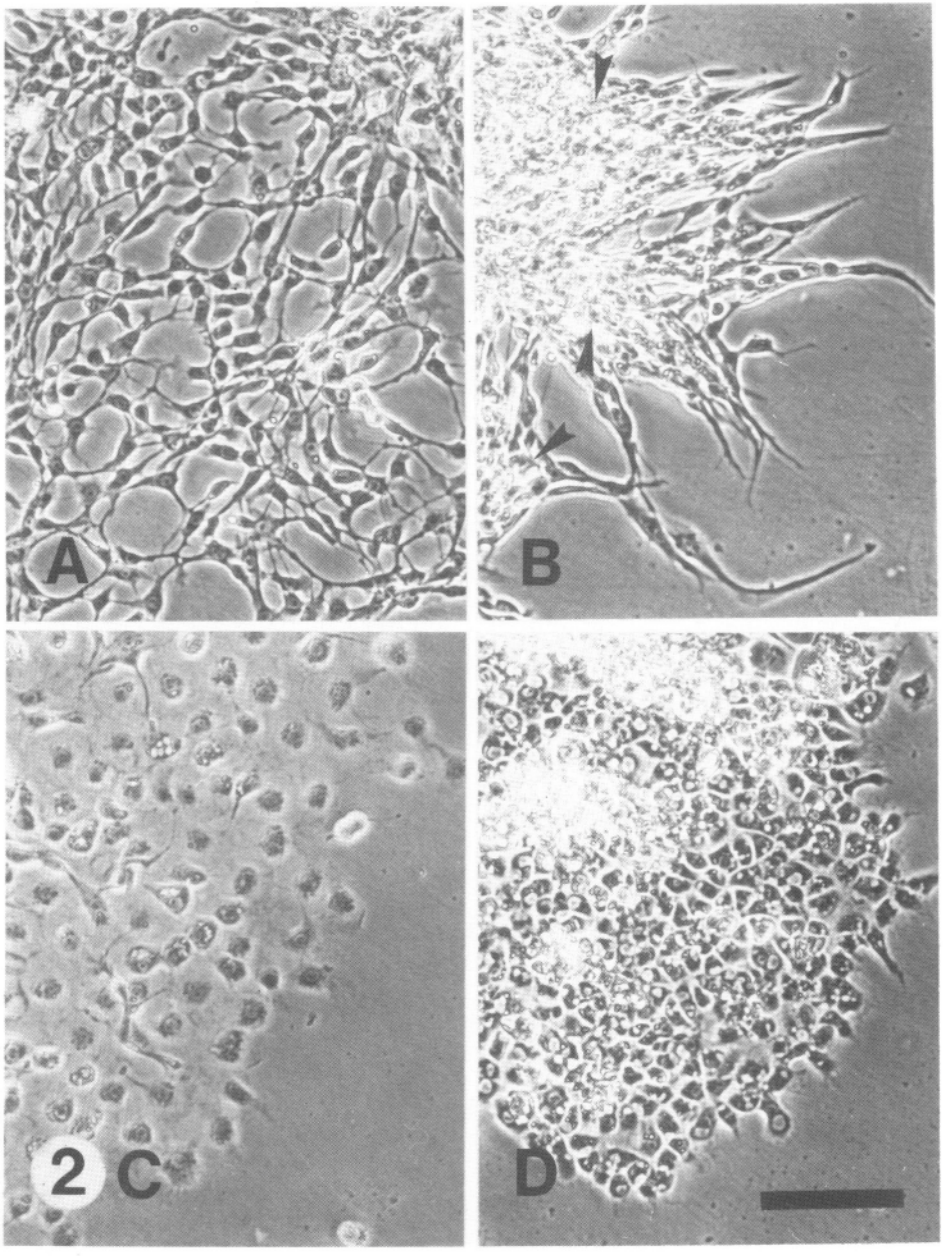

Figure 2. Variability in cell types forming aggregates in an early passage of diamondback moth embryo cells. The presence of such colonies suggest various cell strains can be isolated from a single primary culture of insect embryos. A: Neuroblast-like cells. B: Myoblast cells (cell aggregates indicated by arrow were actively pulsating). C and D: Epithelial-like cell colonies with different cell sizes and morphologies. Scale bar = 100 μm. (Reprinted from Lynn, 1989.)

means you should only have one parent culture, a new flask(s) (already labeled with the cell's identity), one bottle of medium (and a container with the enzyme if you are using one), and a pipettor and pipet in the hood at one time. Any additional objects could affect the air movement in a laminar flow hood and are unnecessary.

In the process of subculturing, as I mentioned above, you should prelabel the new culture flask prior to putting cells into it. The best method is to keep a log

of the subculture procedure in a notebook. When you write in it what you plan to do with the parent culture, for example, in setting up two new cultures of TND1 cells with TNM-FH medium you would write:

20 Sept. 94

Split culture A of passage 29 of TND11:10
with TNM-FH (7 Sept. 94)

new cultures = TND1–30A and –30B

you would also write on the two new culture flasks:

20 Sept. 94 20 Sept. 94

TND1-30A TND1-30B

before you put them under the hood. This procedure should avoid improper labeling of a culture after it has cells in it. You can (and should) compare how a new flask is labeled with the parent flask as you add the cells.

The next rule concerns pipets. It is best to use single-use, disposable pipets, but whichever type of pipet you use, you should *never* use a pipet to go into a bottle of medium twice. This rule will avoid the possibility of accidentally contaminating the medium with the cells. If you use reusable pipets, they should be washed with detergent, thoroughly rinsed with demineralized water and sterilized by autoclaving (at least 121 °C, 151b pressure for 15 min) or dry heat (180 °C for 2 hr). Of course, since we have already determined we are never going to use one bottle of medium for two cell lines, this rule of only using a pipet once might seem extraneous, but it is a very good backup rule to follow. And, of course, we **never** mouth pipet. Use a rubber bulb or one of the mechanical pipettors. The major source of microbial contamination in cell cultures is not the medium or the serum, it is the laboratory worker!

The above covers some of the common mistakes made by new cell culturists. For more extensive information on general procedures for cell culture, see Freshney (1987) or Griffiths *et al.* (1992). These books were written primarily about vertebrate cell culture, but most of the procedures are similar to those used in insect cell culture. Also, for specific techniques on insect cells and tissues which may not be covered in the rest of this volume, see Hink (1989).

Characterization of cell lines

Historically, cell lines have been characterized by morphology and karyology as being a specific cell type or from a particular species. However, cell morphology alone has never been sufficient for characterizing cells. This is because changes in general morphology can occur under different conditions and with time in culture. Karyology is more reliable except for certain cells. Unfortunately, lepidopteran insects are one of the exceptions. Most cell lines from Lepidoptera are highly polyploid and made up of small chromosomes which are impossible to properly karyotype. Better chromosome spreads can be obtained by not using colchicine or colcemid (Disney & McCarthy, 1982), but I recommend using a molecular technique for identifying cells. While DNA fingerprinting may ultimately be a useful technique for this purpose, little effort has been made thus far to determine minimum numbers of probes required for this procedure to be reliable. The isoenzyme technique has been analyzed for use with insect cells (Greene *et al.*, 1972; Tabachnick & Knudson, 1980; Brown & Knudson, 1980, 1982).

The use of isoenzymes relies on the fact that, while organisms have many shared enzyme systems, the particular enzyme protein from a specific organism may differ from other, even closely related, organisms. Thus the protein which acts as the catalyst for converting glucose 6-phosphate to glucose 1-phosphate (phosphoglucomutase, PGM) may be made up of different amino acids in insect A as compared to insect B. These differences can be discerned through electrophoretic techniques.

The electrophoretic method used is not particularly important. Greene and coworkers (1972) used polyacrylamide gels while Knudson's group initially used starch gels (Tabachnick & Knudson, 1980), but later reported that cellulose acetate was a more reliable method (Brown & Knudson, 1980, 1982). Since those reports, a system has been developed commercially (the Authentikit™, Innovative Chemistry, Inc., Marshfield, MA) which uses preformed agarose gels, thus eliminating a major problem with this technique of obtaining consistent results between different gels. Although the reaction buffers needed for staining for the enzymes can be prepared from scratch (see Brown & Knudson, 1980), Innovative Chemistry, Inc. also supplies the reaction buffers as lyophilized powders. While the Authentikit™ is sold with reaction buffers for eight particular enzymes, Tabachnick & Knudson (1980) determined four enzyme systems were

sufficient for discriminating 16 different lines to the species. These enzymes, PGM, phosphoglucose isomerase (PGI), malic enzyme (ME) and isocitrate dehydrogenase (ICD) were subsequently used by Brown & Knudson (1980, 1982) to discern, to the species level, 14 lepidopteran, 20 dipteran and a tick cell line. I have adopted these same four enzymes to characterize cell lines used in my laboratory, an example of which is shown in Figure 3.

Since I use the procedures as outlined in the manufacturer's instructions, I will only provide a brief summary here (all solutions mentioned are obtained from Innovative Chemistry, Inc.). A nearly confluent 25 cm^2 culture flask of cells is suspended by the normal subculture method, transferred to a centrifuge tube and placed on ice. The cells are centrifuged (100 Xg, 5 min[3]), washed once in cold PBS and then recentrifuged. The resulting cell pellet is suspended in extraction buffer, the cells lysed, and centrifuged (800 Xg, 10 min). The resulting supernatant is mixed with an equal volume of stabilization buffer and stored at –20 °C until electrophoresis. One μl of this mixture (or a dilution of the mixture if enzyme activity is too high) is applied to an agarose gel, electrophoresed 25 min at 160 V at 4–10 °C and then stained with the individual reaction buffer at 27 °C[4] for 20–40 min. The gels are washed with distilled water to remove excess reaction buffer, dried and kept as a permanent record of the cell's isoenzyme pattern.

In addition to identification, cell lines need to be periodically screened for contaminants. The primary way to avoid bacterial contamination is by **not** using antibiotics in maintaining cell lines. While this may seem contradictory, the reasoning is simple. If you do not have antibiotics in the medium, any bacterial (or fungal) contamination will become apparent in the highly nutritious cell culture medium within a few days. This will allow you to return to a backup culture to recover the cells. Alternatively, with antibiotics, you may passage the cells for weeks or months with a low level contamination which will eventually become apparent when antibiotic resistance develops in the contaminant. By that time, all your cultures will be contaminated and there will be little hope of recovery. For this reason, I reserve antibiotics for use in "deadend" experiments (experiments in which the cells will no longer be used for maintaining a culture) and for primary cultures. In the case of primary cultures, once regular growth is obtained, I replace the medium being used on the cultures with antibiotic-free medium (usually by the 5th passage).

So, since this avoids most bacterial contamination, our main concern is with viruses and mycoplasma. Here again, avoiding the problem is the best solution. Never use mouth pipetting and obtain your culture supplies (medium, serum, cultureware) from a reputable dealer. One practical advantage of working with insect cells is that many of the contaminants vertebrate cell culturists have to contend with are not an issue with insect cells. For example, since the major source of mycoplasma is the lab worker, these organisms are adapted to grow at 37 °C. The temperatures at which insect cells are grown is not conducive to very effective growth of these organisms (in fact, the insect cells will usually outgrow the bacteria). In the case of viruses, the major source of contamination is serum. Since this is usually of bovine source, these often will not replicate in the insect cell. However, it is still a good idea to periodically screen your cultures for these contaminants.

In the case of mycoplasma, a number of tests are available. These include growth assays using mycoplasma culture medium (such as Mycotrim™, Hana Media, Inc., Berkeley, CA), screening with fluorescent nuclear dyes (such as Hoechst 33258, see Chen, 1976) or coculture with 6-methylpurine (Mycotect, BRL, Bethesda, MD) which is metabolized by mycoplasma to form toxic components. Of these, the Hoechst 33258 method seems the most reliable, but does require a fluorescent microscope. In addition, there are commercial testing facilities which will screen your cultures for mycoplasma (e.g. Flow Laboratories, McLean, VA, and Microbiological Associates, Rockville, MD). Screening for viruses can only be effectively accomplished with an electron microscope, since these are internal contaminants. This is a complicated technique which obviously cannot be covered in detail here, but what you are looking for is any sign of regular arrays of particles.

As mentioned previously, the best solution to contamination is prevention. In the event you do find your cultures are contaminated, it is best to simply discard them and revert to your frozen stock. For this reason, it is very important that you prepare a frozen stock of any new cell lines as soon as possible. The procedure described in Freshney (1987) is similar to the

[3] The centrifugation speed listed here are somewhat lower than that recommended by the manufacturer, but are used because of limitations of my equipment. These have been adequate for obtaining good results with the Authentikit™ system.

[4] The manufacturer recommends 37 °C. The lower temperature cited here is used to be compatible with the insect cell enzymes.

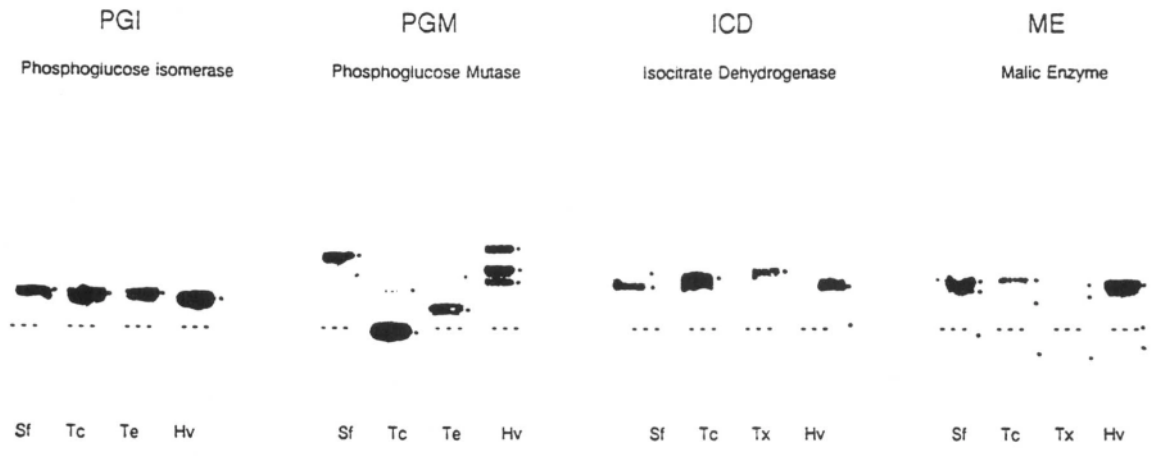

Figure 3. Isoenzyme patterns obtained with various cell lines and staining for four enzymes; PGM, PGI, ICD, and ME. Cell lines shown are IPLB-Sf21AE (Sf, Vaughn *et al.*, 1977), IPLB-Tcon1 (Tc, Lynn and Hung, 1991), IPLB-Tex2 (Te, Lynn and Hung, 1991) and IPLB-HvT1 (Hv, Lynn *et al.*, 1988). The dashed lines mark the location of the origin (where cell extracts were applied prior to electrophoresis) and the small dots to the right of each band were applied with a pen to mark the migration distance. In some cases, weaker bands seen on the gel may not be apparent on the photoreproduction.

method I use. Briefly, cells are placed in suspension by the normal subculture procedure and centrifuged (50 Xg, 10 min). Resuspend the cells in medium containing a cryopreservant. Researchers have used 5–10% dimethyl sulfoxide, but for most insect cells, I prefer 5–10% glycerol. It is best to freeze a few ampules to test the suitability of the cryopreservant prior to making a major freeze for stock purposes. Dispense the cell suspension into 1- or 2-ml glass ampules (Bellco Glass, Vineland, NJ) and seal with a gas/air or gas/O_2 torch. Sealing ampules requires care because improperly sealed vials may inspire liquid nitrogen during storage and will explode during thawing. (Plastic cryovials are also available from several manufacturers, but these also require careful use since they should **never** be used in the liquid phase of LN2.) Sealing ampules should be practiced prior to making a critical freezing of cells. A useful safety technique to test for a good seal is to submerge the sealed ampules in a container of 1% methylene blue in 70% ethanol at 4 °C for 10 min. Any improperly sealed ampules will contain the dye. After sealing, ampules are cooled to freezing. While there are specially designed devices for this, a useful alternative is to place the sealed ampules in a styrofoam box (such as used in shipping chemical supplies) and place it in a –70 °C mechanical freezer. After at least 2 hr at –70 °C, the cells are transferred to a liquid nitrogen freezer (such as Linde freezers, Union Carbide Corp., Indianapolis, IN). An accurate freezer log must be maintained as to the cell line designation and passage number, date of freeze, location in freezer, type of medium, and type/amount of cryoprotectant.

Recovering cells should be done rapidly. A face shield or protective goggles must be worn. This is a precaution for the possibility that the ampule has taken up liquid nitrogen which would cause a dangerous explosion. The ampule is removed from the freezer and placed in warm water (37 °C is usually recommended for vertebrate cells, but I use 30–32 °C to avoid causing a heat shock response which can occur with some insects at 37 °C). As soon as the medium is thawed, wipe the ampule with 70% ethanol, break it open at the neck (scoring the glass with a file if necessary). Transfer the contents to a flask and slowly add 10 ml fresh medium. The cells may be centrifuged at this point and resuspended in fresh medium or left to attach to the flask prior to removing the medium containing the cryopreservant. While initial subcultures following thawing may need to be made at a higher split ratio than before freezing for a few passages, it should be possible to maintain the cells in essentially the same manner as before freezing.

Conclusions

With the wide availability of insect cell culture media, it can generally be considered a routine process to develop new cell lines. Exceptions to this statement do exist, of course. Difficulties may arise when attempting

to culture a specific cell type. For example, while there are a few cell lines from insect fat body and at least one from the midgut, it may not be possible to obtain cell lines from these tissues from all insect species due to terminal differentiation and other factors. Also, researchers have desired cell lines from certain species, such as the honey bee, for which no success has been obtained. As in the early days of tissue culture, it is difficult to discern why negative results occur. However, as more is learned about the physiology and nutrition of various insects and tissues, we may get clues which will help solve these questions.

The remaining chapters in this book will provide the reader with exciting uses for insect cell culture. As I mentioned earlier, the baculovirus expression vector system has provided a stimulus to the field of insect cell culture not seen previously.

References

Altman PL (1961) Hemolymph. Dittmer DS (ed), Blood and Other Body Fluids. (pp. 263–290). Federation of American Societies for Experimental Biology, Washington, DC.

Brown SE & Knudson DL (1980) Characterization of invertebrate cell lines. III. Isozyme analyses employing cellulose-acetate electrophoresis. In Vitro 16: 829–832.

Brown SE & Knudson DL (1982) Characterization of invertebrate cell lines. IV. Isozyme analyses of Dipteran and Acarine cell lines. In Vitro 18: 347–350.

Chen TR (1976) Microscopic demonstration of mycoplasma contamination in cell cultures and cell culture media. TCA Manual 1: 229–232.

Disney JE & McCarthy WJ (1985) A modified technique for the improved characterization of lepidopteran chromosomes from cells in culture. In Vitro Cell. Dev. Biol. 21: 563–568.

Eide PE (1975) Establishment of a cell line from long-term primary embryonic house fly cell cultures. J. Insect Physiol. 21: 1431–1438.

Freshney RI (1987) Culture of Animal Cells: A Manual of Basic Technique. (Second Ed.) New York: Alan R. Liss, Inc.

Gardiner GR & Stockdale H (1975) Two tissue culture media for production of lepidopteran cells and nuclear polyhedrosis viruses. J. Invertebr. Pathol. 25: 363–370.

Grace TDC (1962) Establishment of four strains of cells from insect tissue grown in vitro. Nature 195: 788–789.

Grace TDC (1966) Establishment of a line of mosquito *(Aedes aegypti* L.) cells grown in vitro. Nature 211: 366–467.

Greene AE, Chamey J, Nichols WW & Coriell LL (1972) Species identity of insect cell lines. In Vitro 7: 313–322.

Griffiths JB, Doyle A & Newell DG (eds) (1992) Cell and Tissue Culture: Laboratory Procedures. West Sussex, England: John Wiley & Sons Limited.

Hackett KJ & Lynn DE (1995) Cell culture approach in cultivating spiroplasmas. S Razin & JG Tully (eds), Methods in Mycoplasmology, New York: Academic Press.

Hink WF (1980) The 1979 compilation of invertebrate cell lines and culture media. E. Kurstak, K Maramorosch & A Dübendorfer (eds), Invertebrate Systems In Vitro, (pp. 533–578). Amsterdam: EIsevier/North-Holland Biomedical Press.

Hink WF (1972) A catalog of invertebrate cell lines. In: Vago C, Invertebrate Tissue Culture. (pp. 363–387). New York: Academic Press.

Hink WF (1976) The second compilation of insect cell lines and culture media. Invertebrate Tissue Culture Research Applications (pp. 319-369) New York: Academic Press.

Hink WF (1989) Invertebrate Cell Cultures. J. Tissue Culture Methods 12 (entire issue).

Hink WF & Bezanson DR (1985) Invertebrate cell culture media and cell lines. Tech. in Life Sci. pp. 1–30. County Clare, Ireland: Elsevier.

Hink WF & Hall RL (1989) Recently established invertebrate cell lines. In: Mitsuhashi J, Invertebrate Cell System Applications. Vol. II: (pp. 269–293). Boca Raton, FL: CRC Press, Inc.

Lynn DE (1989) Methods for the development of cell lines from insects. J. Tissue Culture Methods 12: 23–29.

Lynn DE (1989) A technique for isolation of single cell clones from an insect fat body cell line. In Vitro Cell. Dev. Biol. 25: 47A.

Lynn DE, Dougherty EM, McClintock JT & Loeb M (1988) Development of cell lines from various tissues of Lepidoptera. Kuroda Y, Kurstak E & and Maramorosch K (eds), Invertebrate and Fish Tissue Culture. (pp. 239–242). Tokyo: Japan Scientific Societies Press.

Lynn DE & Hung ACF (1991) Development of continuous cell lines from the egg parasitoids *Trichogramma confusum* and *T. exiguum.* Arch. Insect Biochem. Physiol. 18: 99–104.

Nelson-Rees WA, Daniels DW & Flandermeyer RR (1981) Cross-contamination of cells in culture. Science 212: 446–452.

Tabachnick WJ & Knudson DL (1980) Characterization of inverte--brate cell lines. II. Isozyme analyses employing starch gel electrophoresis. In Vitro 16: 392–398.

Vaughn JL, Goodwin RH, Tompkins GJ & McCawley P (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro 13: 213–217.

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New approaches to insect tissue culture

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Key words: insect tissue culture, cell-specific lines, survival, growth, cell differentiation, applications

Introduction

The first insect cell line was established over 30 years ago from the physical dissociation of immature ovaries from a moth (Grace, 1962). Many other insect cell lines containing one of two principle cell types, haemocytes or fibroblasts, have been established since 1962, using similar techniques (Echalier, 1980; Hink, 1980; Hink & Hall, 1989). The methods used in developing insect cell lines have been described in several reviews (Hink & Bezanson, 1985; Lynn, 1989; Vaughn, 1985) and they closely resemble those used in mammalian cell culture (Freshney, 1994). Several types of immature insect tissue have been used to initiate primary cultures including embryos, neonate larvae, imaginal disks and ovaries (Vaughn, 1985). The establishment of cell lines using immature or undifferentiated tissue has been more successful than with mature or differentiated tissues because of the higher proportion of stem cells.

Many researchers have attempted to establish insect cell lines from mature tissue, but with limited success (Mitsuhashi & Shozawa, 1985; Sohi, 1971). Development of cell lines from mature tissue instead of immature tissues has several advantages. First, the identity of the cell types present in the primary cultures is less diverse than immature tissue. Second, it is easy to identify the cell types in mature tissue as the cells have distinct morphologies while, the cells in immature tissues do not. Third, the distinct morphologies allow for assessments of the impact of culture components on the survival of specific cell types in primary cultures. Fourth, changes in cell morphology observed during the transformation process to an immortal cell could provide insight into the identity of the cell types in the cell line. Fifth, marker proteins identified in similar mammalian cells (Freshney, 1992) may be useful for establishing the identity of the the cells in the insect cell line.

Regardless of the source tissue, mature or immature, the initiation of a primary culture starts with the physical and/or enzymatic dissociation of the tissue to produce tissue fragments or cell suspensions. The resulting primary cultures are subjected to a period of "benign neglect" (Vaughn, 1985) to facilitate adaptation to the new environment. Cells undergo a morphological transformation during the first two months in culture which corresponds with the initiation of cell growth (Freshney, 1992). Establishment of a continuous cell line results from a change in cell growth rate referred to as an *in vitro* transformation.

Insect cell culture methods have inadvertently selected for the survival of two cell-specific lines, haemocyte and fibroblast. The only successful attempts to develop cell-specific lines are with insect haemocytes (Mitsuhashi & Shozawa, 1985; Sohi, 1971) and midgut epithelial cells (Baines *et al.*, 1994) that were easy to obtain from the insect in a pure form. Development of cell-specific lines would have several advantages over continuing to produce the same types of insect cell lines using current culture techniques. First, cell-specific lines can be manipulated to express *in vivo* phenology. Second, these altered cells would be extremely useful for studying cell physiology. Third,

cell-specific lines could provide model systems for examining tissue function. Finally, cell-specific lines may expand on the use of insect cell lines as producers of recombinant proteins and bio-insecticides.

The purpose of this paper is to: 1) describe changes to traditional insect cell culture methods that can facilitate the development of cell-specific lines, 2) to describe changes to the culture environment that enhance cell survival and growth, 3) to describe factors that could initiate cell differentiation in insect cell lines, and 4) to describe the potential applications for cell-specific lines.

Development of cell-specific insect cell lines

Development of cell-specific lines from immature tissue has not been a priority in insect cell culture, but there are, however, examples of cell-specific lines developed from mature tissue, including midgut epithelial cells (Baines *et al*., 1994) and haemocytes (Mitsuhashi & Shozawa, 1985; Sohi, 1971). Insect physiologists have maintained a number of different cell types in short-term cultures including nerve (Steele, 1993), muscle (Baines & Downer, 1991), integument (Hiruma & Riddiford, 1989), Malpighian tubule (Coast, 1989), salivary gland (House & Ginsburg, 1979), fat body (Downer, 1979) and haemocytes (Baines *et al*., 1992). The successful use of these short-term cultures to examine physiological processes indicates that the isolation of specific cell types from mature tissues is feasible.

Early failures reported for the development of cell lines from mature insect tissue may have resulted from inappropriate dissociation procedures. Trypsin is the most popular enzyme used to dissociate immature tissues, but other enzymes have also been employed including collagenase, dispase and hyaluronidase (Vaughn, 1985).Whole or portions of immature tissue are exposed to trypsin at 25–37 °C for a brief time period (Kurtti & Brooks, 1977; Quiot *et al*., 1985; Sohi, 1973). Dissociation of mature lepidopteran tissues with trypsin at 13–25 °C for 1–16 hr, did not provide healthy cells (Baines, unpubl.) whereas, we were able to obtain healthy midgut epithelial cells by dissociating midgut in collagenase at 13 °C for 16 hr (Baines *et al*., 1994). This sensitivity of mature tissue to trypsin has also been observed for some mammalian tissues and it has been replaced with gentler enzymes, such as collagenase and dispase (Freshney, 1992). The first key requirement for initiating a cell line from mature tissue is to select an enzyme and the appropriate conditions for tissue dissociation.

The early difficulties encountered culturing cells from mature tissue (Vaughn, 1985) may also be attributable to the consistent use of mixed cell populations in primary cultures.

Once immature tissues have been dissociated, the mixed cell suspension is used to initiate a primary culture. The cell profile changes in the primary culture as the cells transform to an immortal state (Mitsuhashi & Shozawa, 1985; Sohi, 1973). There is some indication in the literature that the presence of several cell types in primary cultures could contribute to the death of specific cell types. Insect cell lines and immature tissues can produce the hormone, 20-hydroxyecdysone (Loeb *et al*., 1991b; Lynn *et al*., 1987; Ward *et al*., 1987). This hormone initiates developmental processes in immature tissues (Loeb, 199la,b) and insect cell lines (Berger *et al*., 1980; Cherbas *et al*., 1980; English *et al*., 1987; Oberlander *et al*., 1987) that ultimately leads to cell death. Primary cultures established with cells from mature tissue could also produce similar compounds that selectively kill susceptible cells. It has been shown that primary cultures from whole midgut (muscle, epithelial cells, connective tissue, nerve) preparations die after terminal cell differentiation in 10 weeks (Sadrud-Din *et al*., 1992) whereas, removal of the muscle/connective/nerve tissue from the cell preparation permitted the development of midgut epithelial cell lines (Baines *et al*., 1994).

A second key requirement for initiating primary cultures from mature tissues is to develop methods that separate cell types from the cell suspension obtained from the tissue. Some mature insect cells are easy to obtain in pure form (Sohi, 1971), while most cells are isolated by taking advantage of tissue features, such as differential susceptibility to enzyme (Baines *et al*., 1994) and cell density (Freshney, 1992). Baines *et al.* (1994) removed epithelial cells from the surrounding muscle/connective tissue layer to obtain the cells necessary to initiate primary cultures. Examination of each fraction showed that collagenase degraded both the basal lamina and the connections between the midgut epithelial cells, but it did not affect the muscle layer. If a mixed cell suspension is obtained from the dissociation of tissue, they can be separated on the basis of cell density using simple density gradients, such as Percoll and Ficoll. This technique has been used in insect physiology to isolate different haemocyte types (Soderhall & Smith, 1983). In mammalian cell culture, high purity cell suspensions are used to initiate primary

cultures to ensure that a particular cell type survives in the cell line (Freshney, 1992; Chew, 1994). A similar pattern has been observed in the successful development of cell lines from mature midgut epithelial cells (Baines *et al.*, 1994).

Modification of the culture environment

The culture environment is created by the interaction of a variety of components including medium composition, serum factors, serum hormones, culture unit and medium volume. These interactions will ultimately affect the ability of cells to survive and transform into immortal cells. A critical research goal for insect cell culture is to begin experimenting with these components to determine the optimal conditions for maintaining specific cell types in primary cultures.

Medium composition

The majority of insect media have similar formulations in that they contain salts, amino acids, vitamins, sugar, and buffer (Vaughn, 1985). They may also contain undefined components such as tryptose phosphate broth, yeast hydrolysate and lactalbumin hydrolysate (Mitsuhashi, 1989). A simple approach to assessing the impact of these components on specific cell types is to test varying concentrations of medium to support short-term cell survival. This procedure has been used to adapt existing media developed for particular insect families for use with other insect families (Gardiner & Stockdale, 1975, Kurtti & Munderloh, 1989; Shearn *et al.*, 1980).

Medium composition can dictate several general environmental features including pH, osmolarity and Na^+/K^+ ratio. The first feature, pH, varies for haemolymph among and within insect families (eg. cockroach vs. moth) (Pannabecker *et al.*, 1992; Vaughn, 1985). Insect cell lines require the maintenance of a specific range of pH for optimal growth (Sohi, 1980) and there may be a similar requirement for cells in primary culture. Fluctuations in pH are prevented in large scale culture of insect cell lines by adding a second buffer to supplement the bicarbonate buffer already present in the culture medium (Goodwin & Adams, 1980; Quiot *et al.*, 1985). Manipulation of buffers may also prove useful for medium used in primary cultures.

The second feature, osmolarity, also varies for haemolymph among and within insect groups (Vaughn, 1985; Pannabecker *et al.*, 1992). Since insect cell lines tolerate a wide range of osmolarity (Sohi, 1980), it was assumed that insect tissue in primary culture is also tolerant. This may not be true for individual cell types and should be assessed before initiating primary cultures for the development of insect cell lines.

The third feature, the Na^+/K^+ ratio, is either at low or high levels in insect medium and these ratios reflect the degree of cell exposure to insect haemolymph *in vivo* (Mitsuhashi 1989). For example, the insect nervous system is protected from the low Na^+/K^+ ratio of the haemolymph by a layer of glial cells which are selectively permeable to ions and maintain a high Na^+/K^+ ratio within the tissue. Thus, primary cultures of nerve cells require a medium with a high Na^+/K^+ ratio that mimics the normal environment of the functional tissue (Beadle & Hicks, 1985). Ion concentration in the medium can also affect the activity and survival of a cell type in primary culture. Insect muscle has been observed in primary cultures initiated from immature tissue (Rochford *et al.*, 1984; Sohi, 1968). The level of K^+ ions in the low Na^+/K^+ ratio medium causes continuous spontaneous contractions (Aidley, 1985). This cell type was not present in the developed cell lines indicating that the constant activity may not be beneficial to long term cell survival. Thus, it is essential to consider the physiology and structure of a tissue when designing an appropriate environment.

Serum factors

"Complete" medium normally has from 5 to 20% serum that contributes both positive and negative factors for the development of a cell line. The benefit of this medium is the presence of attachment and nutritional factors that facilitate adaptation to the culture environment (Freshney, 1992). The detrimental effect of complete medium stems from the presence of factors that are toxic to cells, such as endotoxin (McIntosh *et al.*, 1976).

The easiest approach to overcoming problems with serum for primary cultures of insect cells would be to stop using it as a medium supplement. This has only recently been accomplished with insect cell lines in large scale culture (Hink & Strauss 1980; Kurtti & Munderloh, 1989; Mitsuhashi & Goodwin, 1989). Removal of serum has caused several changes in the condition and requirements of the cells including increased fragility, sensitivity to low temperatures, and a mandatory requirement for sterols.

The first change, fragility, may be compensated by the addition of compounds that increase the specific gravity of medium (Mitsuhashi & Goodwin, 1989). Starch (Takagi *et al*., 1994) and methylcellulose (Hink & Strauss, 1989) have been added to culture medium to effectively protect cells from sheer stress in large scale culture. The original studies were performed on cells growing in stir flasks where cells are kept in constant motion. It is unlikely that the culture environment provided by a standard culture flask is as harsh as a stir flask, but it may prevent stress induced by medium movement (Aloi & Cherry, 1994) during regular assessments of primary cultures. These agents must be tested for their effects on the survival of insect cells in primary culture.

The second change, sensitivity to lower temperature, need only be taken into account when cells in culture have transformed and they are being prepared for storage in liquid nitrogen (Mitsuhashi & Goodwin, 1989). At that time, cells can be frozen in glycerol-based freezing medium to prevent high cell losses caused by storage and freeze-thawing procedures.

The third change, requirement for sterols, has been achieved by the addition of lipid emulsions to culture medium (Brooks *et al*., 1980; Goodwin, 1985; Goodwin, 1989). This need stems from a lack of biochemical pathways to produce these substances in insects. We have been using a cholesterol-rich lipid supplement (Sigma Chemical Co.) instead of heat-inactivated fetal bovine serum to initiate primary cultures of haemocytes, midgut epithelial cells and fat cells without any apparent impact on cell survival (Baines, unpubl.). In other instances, the addition of yeastolate (Kurtti & Munderloh, 1989) or tryptose phosphate broth (Lee & Park, 1994) to culture medium reduced the level of serum required for growth of insect cell lines. Thus, these additives may prove useful for the development of serum-free insect medium.

Serum hormones

Growth hormones are also provided by serum and they are essential additives of defined media in mammalian cell culture (Freshney, 1992). The beneficial effect of including growth hormones is faster growth and proliferation of cells in either primary or continuous cultures. Insects have two principle growth hormones, 20-hydroxyecdysone and juvenile hormone, that coordinate growth and development (Mordue *et al*., 1980). A great deal of information is available on the effect of 20-hydroxyecdysone on the development of immature tissue and insect cell lines (Porcheron, 1991). 20-Hydroxyecdysone initiates developmental events in primary cultures of integument (Riddiford *et al*., 1980), spermducts (Loeb, 1991a,b), and imaginal discs (Ui *et al*., 1989). It has also been shown to cause cell differentiation (Loeb, 199la) and changes in cell volume (English *et al*., 1984) in insect cell lines. There are several classes of juvenile hormone that apparently maintain larval characters while inhibiting metamorphosis (Mordue *et al*., 1980). Another insect hormone, insulin-like hormone has been shown to promote the establishment and growth of Drosophila cell lines (Ui *et al*., 1989). Thus, juvenile hormone and insulin-like hormone may prove useful as additives to defined medium to promote the growth of cells in primary culture.

There are a number of commercially available growth factors designed for mammalian cell culture (Freshney, 1992). We tested the effect of endothelial growth factor, nerve growth factor and cholera toxin compared with fetal bovine serum on the establishment and growth of fat cells from tissue fragments in primary cultures (Baines, unpubl.). Both growth factors caused an epithelial-like spreading and attachment of the tissue fragments to the culture vessel, but there was no obvious beneficial effect on cell growth. These effects were identical to those observed with 20% fetal bovine serum suggesting that either of these compounds may be useful as additives to insect medium to promote cell attachment. Cholera toxin at 10^{-12} M, caused initial swelling and then, rupturing of the tissue suggesting that insect cells are much more sensitive to this compound than mammalian cells. Other factors have been shown to have a positive effect on insect cell growth. Specific vitamins including choline, biotin, and inositol, were shown to promote cell growth in immature tissues (Mituhashi, 1989) while, the non-physiological compound, pluronic F–68, has been shown to enhance the growth rate of an insect cell line (Lee & Park, 1994). A combination of these factors might be effective in a defined medium to provide a stimulus for cell growth in primary cultures.

Serum can also provide hormones that have a negative effect on the development of a cell-specific line. Mammalian serum contains a fibroblast growth factor that facilitates the survival of this cell type and not others in the final culture (Freshney, 1992). Insect physiologists have identified similar hormones in insects as those observed in mammals (Stanley-Samuelson, 1994) and it is possible that this hormone may have a similar effect on insect fibroblast cells in prima-

ry cultures. Mammalian fibroblasts are controlled in cultures by including inhibitory agents (D-valine, cis-hydroxyproline), seeding cells onto UV-irradiated fibroblast feeder layers, or by using heat-inactivated serum. We have used heat-inactivated fetal bovine serum to establish an epithelial cell line in insects (Baines *et al*., 1994) and this may have been aided by the exclusion of the fibroblast growth factor. The other treatments have not been tested for their impact on the development of insect cell lines.

Culture unit

Cells are organized in a tissue to perform a set of physiological functions and this is often facilitated by interactions with other cells in the tissue (Mordue *et al*., 1980). It is possible to maintain this type of environment without compromising the development of a cell-specific line. Structural supports such as membrane inserts and feeder layers, can recreate the interactive environment of the tissue without allowing the undesirable tissue to overtake the culture. These procedures have been used extensively for mammalian epithelial cells and they promote the growth of specific cell types (Fusenig, 1992). This technology has not been adapted for use in the development of insect cell lines.

Medium volume

Insect tissues are normally supplied with oxygen by trachea (Mordue *et al*., 1980) and the density of this tissue reflects a tissue's oxygen requirements. Oxygen has been shown to play a pivotal role in the growth of insect cell lines in large scale culture (Deutschmamn & Jager, 1994; Hink & Strauss, 1989; Wang & Bentley, 1994) and in maintaining the normal metabolic activity of tissue *in vitro* (Noriega & Wells, 1992). It has also been shown that the responsiveness of insect cell lines to hormones is significantly better in the presence of 50% oxygen than that obtained from cells maintained under standard culture conditions (Courgeon *et al*., 1989). This suggests that part of the slow adaptation to the culture environment for insect cells in primary culture may be a function of oxygen levels. Confirmation of the necessity for higher oxygen levels in primary cultures has been given by researchers establishing cell lines in low volume cultures (Sang, 1980; Sato, 1989). They found that the success rate for establishing cell lines was greater in these cultures.

Low volume cultures are relatively easy to maintain, but there is a greater chance that critical medium components may be used or degraded quickly. One way to overcome this difficulty is to provide greater oxygen levels under standard culture conditions. Noriega & Wells (1992) developed a technique for incorporating oxygen into culture medium using the oxygen-carrying compound, perfluorotributylamine. They determined that the improved oxygen levels enhanced the metabolic activity of fat lobes *in vitro*. We maintained fat body cells in primary cultures using this compound under standard culture conditions and obtained similar cell survival as observed in low volume culture (Baines, unpubl.). This suggests that it may be possible to use these types of compounds to promote the establishment of cell lines from oxygen-demanding cells.

Fibroblasts and haemocytes do not have a direct oxygen source *in vivo*. The predominance of these cell types in insect cell lines supports the view that current culture techniques do not supply enough oxygen to allow other oxygen-demanding cells to survive in primary cultures. It is essential to develop methods to enhance the level of oxygen in the culture environment to promote the survival of other cell types.

Characterization of cell types

Insect cell lines have been characterized to insect family, but not cell type. This has been accomplished by determining isozyme and/or karyotype profiles (Greene *et al*., 1972; Harvey & Sohi, 1989). The other information provided for insect cell lines include descriptions of morphology and growth rate (Vaughn, 1985). Two factors have contributed to the limited use of insect cell lines: first, the unclear origin of cell types dictates that only general phenomenon can be examined and second, our inability to create a functional differentiated cell type prevents any correlation with *in vivo* processes.

There are several approaches that could be taken to identify cell types present in a cell line. First, changes in cell morphology could be documented throughout the development of the cell line using electron microscopy. This approach has recently been used to follow the transformation of insect cells in primary cultures (Kuroda & Shimada, 1989) and can easily be expanded to follow the changes in cells during development to a continuous cell line. Second, there may be unique proteins in a cell that could be used as a marker. In mammalian cell culture, cell types are distinguished by the presence of unique membrane and structural proteins (Chew, 1994; Evans *et al*., 1994).

Several marker proteins have been identified in mammalian epithelial cells including intermediate filament proteins, desmosome junctions, and cell surface antigens (Freshney, 1992). This information will be used to confirm the presence of epithelial cells in the cell lines developed from midgut epithelial cells (Baines *et al*., 1994). Third, monoclonal antibodies have been produced from proteins in insect cells (Oddou *et al*., 1993) and these methods can be applied to insect cell culture to produce antibodies that recognize specific cell types. A similar approach has been taken to confirm the identify of the insect family that contributed the tissue for developing a cell line (Vaughn *et al*., 1977).

Factors promoting cell differentiation

There are two distinct cell sources that could serve as the basis for developing model *in vitro* cultures for the study of differentiated cell functions; the first source is existing insect cell lines with a limited variety of cell types while, the second is new cell-specific lines. In either case, a method has to be developed to affect the balance between stem, precursor and differentiated cells. In mammalian cell culture this balance is affected by several factors: 1) soluble physiological and non-physiological inducers, 2) cell-cell interactions and 3) cell-matrix interactions (Freshney, 1992).

There are three physiological factors that stimulate cell differentiation in mammalian systems including hormones (hydrocortisone/insulin), a vitamin (retinoic acid) and an ion (calcium). The most likely insect-equivalent for the hormones are 20-hydroxyecdysone and insulin-like hormone. 20-hydroxyecdysone causes the differentiation of immature tissues (Loeb, 1991 a,b) and insect cell lines (Cherbas *et al*., 1980) while, insulin-like hormone caused the differentiation of some *Drosophila* cell lines (Ui *et al*., 1989). It may also be useful to test other mammalian hormones for their effects on insect cell lines.

The second physiological factor, retinoic acid has not been examined for its effect on cell differentiation in insect cell lines, but it did not affect the growth of a cell line (Mitsuhashi & Goodwin, 1989). Further tests are necessary to evaluate the ability of this compound to elicit responses from insect cell lines.

The third physiological factor, calcium ions, differs dramatically between the two principal types of media used to culture insect tissues, high Na^+/K^+ ratio (26) and low Na^+/K^+ ratio (0.2) medium (Mitsuhashi, 1989). Low Na^+/K^+ medium has a 5× greater concentration of calcium ions than high Na^+/K^+ medium. It is not known if the increased level of calcium ions contributes to survival of specific insect tissues in the different media.

Several non-physiological factors can cause the differentiation of mammalian cell lines including sodium butyrate, dimethyl sulphoxide, hexamethylenebisacetamide, and n-methyl- ordimethyl-formamide (Freshney, 1992). These materials have not been tested for their ability to affect insect cell lines.

The remaining factors that initiate cell differentiation are cell-cell interactions and cell-matrix interactions. Cell-cell interactions can facilitate the differentiation of cells to form functional tissues in mammalian cell culture (Freshney, 1992). The development of spermducts occurred in the presence of fat body, but not alone *in vitro* (Loeb, 1991a). This suggests that insect tissues are induced to differentiate in the presence of other tissues. Special culture chambers are available in mammalian cell culture for separating cells from each other in the same culture unit (Fusenig, 1992). The commercial availability of membrane inserts (Costar Corporation, Toronto) and gels that have low gelling temperature, Sea Plaque (Mandel Scientific Company, Quebec) provide the necessary tools to test the potential benefits of coculturing different insect cells in primary culture. Cell:matrix interactions are achieved by coating culture dishes with one or more matrix components including collagen, laminin, heparin, fibronectin, poly-D-lysine, and vitronectin (Freshney, 1992). Mammalian epithelial cells will attach to a surface coated with matrix materials forming a functional epithelium. We were unable to initiate cell-matrix interactions using similar procedures with midgut epithelial cells (Baines *et al*., 1994). It is difficult to interpret this result as it is unclear whether the culture environment is suppressing the normal activity of midgut epithelial cells.

Another approach for creating an environment for cell differentiation is to seed cells onto cell feeder layers. Mammalian epithelial cells form a functional tissue in response to the hormones released from fibroblasts present in a feeder layer (Fusenig, 1992). It may be possible to use fibroblast-like insect cell lines in a similar organotypic culture system to promote the differentiation of cell-specific lines.

Use of cell-specific continuous cultures

There is a great deal of information available on the regulation of whole tissue activities in insects (Downer & Hiripi, 1993). The corresponding cells responsible for the dominant tissue function have also been isolated for physiological studies using physical or enzymatic procedures (see above). There are two problems with this approach; first, the short-term viability of the cells and second, the uncertainty that the tissue reflects *in vivo* activities (Evans *et al*., 1994). The availability of cell lines that could be manipulated to form functional tissues would greatly enhance the use of insect cell lines in insect physiology.

There are a number of applications for cell-specific insect cell lines. There is a need to establish the impact of bio-insecticides on target cells in pest insects and non-target insects (Johnson, 1994; Stipanovic *et al*., 1990). We are using primary cultures of midgut epithelial cells from several forest pests to determine the mode of action of Bt toxins. The use of primary cultures is a labor intensive process requiring a constant supply of insects and preparation of tissue. The availability of midgut epithelial cell lines from our forest target insects will greatly enhance our ability to assess the effects of bio-pesticides. In addition, any cell line developed from the first target tissue of bio-insecticides, midgut epithelial cells, would be useful for characterizing cell homeostasis mechanisms which may be useful model systems for developing novel bio-insecticides (Fusenig, 1992)

Insect cell lines have been shown to secrete insect hormones (Loeb *et al*., 1989; Lynn *et al*., 1987) and other novel compounds (Okai, 1985) with potential commercial applications. A greater diversity of insect cell lines could provide a better opportunity for isolating other novel compounds.

Isolation and extraction of specific proteins from insects has been hampered by the time and labour necessary to isolate even small quantities of material (Lange *et al*., 1993). The creation of cell lines that expressed a desired protein could greatly speed up this process.

Characterization of insect receptors is achieved by preparing membrane preparations of a cell type. Only one receptor type has been located in insect cell lines, octopamine (Gole *et al*., 1982), and the expression of the receptor declines with subsequent passages. Thus, the availability of specific cell lines that can be manipulated to express the normal spectrum of receptors would provide a valuable model system to study the receptor-mediated events affecting cellular activity *in vivo*.

Baculovirus have proven useful as both bio-insecticides and as agents facilitating the expression of foreign protein in insect cells (Davis *et al*., 1993; Gelernter & Federici, 1986). In both cases, the Sf–9 cell line has been used most extensively. A major hurdle to the development of baculovirus bio-insecticides is the lack of permissive cell lines from target insects (Granados & Hashimoto, 1989). This could be overcome in the early stages of investigation by assessing the permissiveness of several cell types from the target insects in primary culture. The cell type that provides the best virus yield and pathogenic properties, can be used to initiate both primary cultures for the development of a cell line and studies of the molecular biology and genetics of the virus.

Conclusion

Current methods of insect cell culture have produced a limited variety of cell types in an ever expanding list of insect cell lines. In developing midgut epithelial cell lines, we found that traditional methods in insect cell culture failed to provide healthy cells from mature tissues. Examination of mammalian cell culture literature for this particular cell type provided the insight required to successfully develop a cell-specific line (Baines *et al*., 1994). The potential applications for cell-specific lines from insects are numerous. This paper is a compilation of ideas that will hopefully enable other researchers to develop additional cell-specific lines.

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References

Aidley DL (1985) Muscular contraction. In: Kerkut GA & Gilbert LI (eds) Comprehensive Insect Physiology Biochemistry and Pharmacology. Vol. 5 (pp. 407–438), Pergamon Press, New York.

Aloi LE & Cherry RS (1994) Intracellular calcium response of Sf–9 insect cells exposed to intense fluid forces. J. Biotechnol. 33: 21–31.

Baines D, Brownwright A, Schwartz J-L (1994) Establishment of primary and continuous cultures of midgut epithelial cells of the spruce budworm, gypsy moth and silkworm. J. Insect Physiol. 40: 347–357.

Baines D, Desantis T & Downer RGH (1992) Octopamine and 5-hydroxytryptamine enhance the phagocytic and nodule formation activities of cockroach (*Periplaneta americana*) haemocytes. J. Insect Physiol. 38: 905–914.

Baines RA & Downer RGH (1991) Pharmacological characterization of a 5-hydroxytryptamine-sensitive receptor/adenylate cyclase complex in the mandibular closer muscles of the cricket, *Gryllus domestica.* Archs. Insect Biochem. Physiol. 16: 153–163.

Beadle DJ & Hicks D (1985) Insect nerve culture. In: Kerkut GA & Gilbert LI (eds) Comprehensive Insect Physiology Biochemistry and Pharmacology. Vol. 5 (pp. 181–211) Pergamon Press, New York.

Berger EM, Frank M & Abell MC (1980) Ecdysone-induced changes in protein synthesis in embryonic *Drosophila* cells *in vitro.* In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 195–208) Elsevier/North-Holland Biomedical Press, New York.

Brooks MA, Tsang KR & Freeman FA (1980) Cholesterol as a growth factor for insect cell lines. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 67–78) Elsevier/North-Holland Biomedical Press, New York.

Chew CS (1994) Parietal cell culture: new models and directions. Annu. Rev. Physiol. 56: 445–61.

Cherbas L, Cherbas P, Savakis C, Demetri G, Manteuffel-Cymborowska M, Younge CD & Williams CM (1980) Studies of ecdysteroid action on a *Drosophila* cell line. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 217–228) Elsevier/North-Holland Biomedical Press, New York.

Coast GM (1989) Stimulation of fluid secretion by single isolated malpighian tubules of the house cricket, *Acheta domesticus.* Physiol. Entomol. 14: 21–30.

Courgeon AM, Ropp M, Rollet E, Becker J, Maisonhaute C, Echalier G & Best-Belpomme M (1989) Relationships between ecdysterone-induced cellular differentiation and aerobiosis in an *in vitro Drosophila* cell system. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 1 (pp. 93–97) CRC Press Inc, Florida.

Davis TR, Wickham TJ, McKenna KA, Granados RR, Shuler ML & Wood HA (1993) Comparative recombinant protein production of eight insect cell lines. In Vitro Cell. Dev. Biol. 29A: 388–390.

Downer RGH (1979) Trehalose production in isolated fat body of the American cockroach, *Periplaneta americana.* Comp. Biochem. Physiol. 62C: 31–34.

Downer RGH & Hiripi L (1993) Biogenic amines in insects. In: Borkovec AB & Loeb MJ (eds) Insect neurochemistry and neurophysiology. (pp. 23–38) CRC press, Ann Arbor.

Deutschman, DM & Jager V (1994) Optimization of the growth conditions of Sf21 insect cells for high-density perfusion culture in stirred-tank bioreactors. Enzyme Microb. Technol. 16: 506–512.

Echalier G (1980) Necessity of radically new insect cell culture methods. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 589–592) Elsevier/North-Holland Biomedical Press, New York,

English LH, Magelky BK & Marks EP (1984) 20-Hydroxyecdysone-induced changes in the cell volume of lepidopteran cells associated with population dynamics. In Vitro 20: 71–78.

Evans GS, Flint N & Potten CS (1994) Primary cultures for studies of cell regulation and physiology in intestinal epithelium. Annu. Rev. Physiol. 56: 399–417.

Freshney RI (1992) Introduction. In: Freshney RI (ed) Culture of Epithelial Cells, (pp. 1–23) Wiley-Liss, New York.

Freshney RH (1994) Culture of animal cells. Wiley-Liss, New York.

Fusenig NE (1992) Cell interaction and epithelial differentiation. In: Freshney RI (ed) Culture of Epithelial Cells. (pp. 25–57) Wiley-Liss, New York.

Gardiner GR & Stockdale H (1975) Two tissue culture media for production of Lepidopteran cells and nuclear polyhedrosis viruses. J. Invert. Pathol. 25: 363–370.

Gelernter WD & Federici BA (1986) Continuous cell line from *Spodoptera exigua* (Lepidoptera: Noctuidae) that supports replication of nuclear polyhedrosis viruses from *Spodoptera exigua* and *Autographa californica.* J. Invert. Path. 48: 199–207.

Grace TDC (1962) Establishment of four strains of cells from insect tissues grown *in vitro.* Nature 195: 788–789.

Gole JWD, Downer RGH & Sohi SS (1982) Octopamine-sensitive adenylate cyclase in haemocytes of the forest tent caterpillar, *Malacosoma disstria* Hubner (Lepidoptera: Lasiocampidae). Can. J. Zool. 60: 825–829.

Goodwin RH & Adam JR (1980) Nutrient factors influencing viral replication in serum-free insect cell line culture. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 493–510) Elsevier/North-Holland Biomedical Press, New York.

Goodwin RH (1985) Growth of insect cells in serum-free media. Techniques in the Life Sciences: Setting Up and Maintenance of Tissue and Cell Cultures C(109): 1–28.

Goodwin RH (1989) Construction of peptoliposomes for the incorporation of nutrient lipid supplements in insect cell culture medium. J. Tissue Culture Methods 12: 17–20.

Granados RR & Hashimoto Y (1989) Infectivity of baculoviruses to cultured cells. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 2 (pp. 3–13) CRC Press Inc, Florida.

Greene AE, Charney J.Nichols WW & Coriell LL (1972) Species identity of insect cell lines. In Vitro 5: 313–322.

Harvey GT & Sohi SS (1989) Isozyme characterization of 8 Hymenopteran and 20 Lepidopteran Cell lines. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 1 (pp. 71–76) CRC Press Inc, Florida.

Hink WF (1980) The 1979 compilation of invertebrate cell lines and culture media. In. Invertebrate Systems in vitro. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 553–578) Elsevier/North-Holland Biomedical Press, New York.

Hink WF & Bezanson DR (1985) Invertebrate cell culture media and cell lines. Techniques in the Life Sciences:Setting Up and Maintenance of Tissue and Cell Cultures. C111: 1–30.

Hink WF & Hall RL (1989) Recently established cell lines. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 2 (pp. 269–293) CRC Press Inc, Florida.

Hink WF & Strauss EM (1980) Semi-continuous culture of the TN-368 cell line in fermenters with virus production in harvested cells. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 27–34) Elsevier/North-Holland Biomedical Press, New York.

House CR & Ginsberg BL (1979) Pharmacology of cockroach salivary secretion. Comp. Biochem. Physiol. 63C: 1–6.

Johnson DE (1994) Cellular toxicities and membrane binding characteristics of insecticidal crystal proteins from *Bacillus thuringiensis* toward cultured insect cells. J. Invert. Pathol. 63: 123–129.

Kuroda Y & Shimada Y. (1989) Electron microscopic studies on *in vitro* differentiated cells from *Drosophila* embryos. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 1 (pp. 77–89) CRC Press Inc, Florida.

Kurtti TJ & Brooks MA (1977) Isolation of cell lines from embryos of the cockroach, *Blattella germanica.* In Vitro 13: 11–17

Kurtti TJ & Munderloh UG (1989) Advances in the definition of culture media for mosquito cells. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 1 (pp. 21–30) CRC Press Inc, Florida.

Lee SH & Park TH (1994) Growth limiting factors influencing high density culture of insect cells in Grace's medium. Biotechnol. Lett. 16: 327–332

Loeb MJ (1991a) Development of isolated spermducts from *Heliothis virescens* (Lepidoptera)*in vitro.* Invert. Reprod. Develop. 21: 67–73.

Loeb MJ (1991b) Growth and development of spermducts of the tobacco budworm moth *Heliothis virescens*, *in vivo* and *in vitro.* Invert. Reprod. and Dev. 19: 97–105.

Loeb MJ, Brandt EP & Woods CW (1989) Testes of the tobacco budworm moth: ecdysteroid production by the testis sheath. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 1 (pp. 105–110) CRC Press Inc, Florida.

Lynn DE (1989) Methods for the development of cell lines from insects. J. Tissue Culture Methods 12: 23–29.

Lynn DE & Oberlander H (1989) Characterization of cell lines derived from imaginal disks of three species of Lepidoptera. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 2 (pp. 207-212) CRC Press Inc, Florida.

Lynn DE, Feldlaufer MF & Lusby WR (1987) Isolation and identification of 20-hydroxyecdysone from a Lepidopteran continuous cell line. Arch. Insect Biochem. Physiol. 5: 71–79.

Mclntosch AH, Evers D & Shamy R (1976) A toxic substance in fetal bovine serum (abstract). In Vitro 12: 302.

Mitsuhashi J (1989) Nutritional requirements of insect cells *in vitro.* In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 1 (pp. 3–20) CRC Press Inc, Florida.

Mitsuhashi J & Goodwin RH (1989) Serum-free cultures of insect cells *in vitro.* In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 1 (pp. 31–14) CRC Press Inc, Florida.

Mitsuhashi J & Shozawa A (1985) Continuous cell lines from larval haemocytes of the cabbage armyworm, *Mamestra brassicae.* Develop. Growth and Differ. 27: 599–60

Mordue W, Goldsworthy GJ, Brady J & WM Blaney (1980) Insect Physiology, Blackwell Scientific Publications, Oxford.

Noriega FG & Wells WA (1992) Oxygen-carrying perfluorochemical emulsions improve insect fat body culture performance. Insect Biochem. Molec. Biol. 22: 585–590.

Oberlander H, Leach CE, Lanka S, & Willis JH (1987) Ecdysteroid action on moth epithelial tissues and cell lines. Arch. Insect Biochem. Physiol. 5: 81–89.

Oddou P, Hartmann H, Radecke F & Geiser M (1993) Immunologically unrelated *Heliothis* sp. and *Spodoptera* sp. midgut membrane-proteins bind *Bacillus thuringiensis* CryIA(b) δ-endotoxin. FEBS 212: 145–150.

Okai Y (1985) Insect fibroblast-like cells release inhibitory peptides of lymphocyte DNA synthesis into serum-free culture medium. Insect Biochem. 15: 483–487.

Pannabecker TL, Andrews F & Beyenbach KW (1992). A quantitative analysis of the osmolytes in the hemolymph of the larval gypsy moth, *Lymantria dispar.* J. Insect Physiol. 38: 823–830.

Porcheron P (1991) Insect tissue culture systems: models for study of hormonal control of development. In Vitro Cell. Dev. Biol. 27A: 479–482.

Quiot JM, Paradis S & Louis C (1985) Establishment of a cell line (SPC Gl 13) from dorsal vessels of *Gromphadorhina laevigata*, *saussure*, and *zentner* (Dictyoptera: Blattidae). In Vitro Cell. Develop. Biol. 21: 603–605.

Riddiford LM, Kiguchi K, Roseland CR, Chen AC & Wolfgang WJ (1980) Cuticle formation and sclerotization *in vitro* by the epidermis of the tobacco hornworm, *Manduca sexta.* In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 103–117) Elsevier/North-Holland Biomedical Press, New York.

Rochford R, Dougherty EM & Lynn DE (1984) Establishment of a cell line from embryos of the cabbage looper, *Trichoplusia ni.* In Vitro 20: 823–825.

Sadrud-Din S, Hakim RS & Loeb M (1992) Establishment of a midgut cell culture from Lepidoptera (abstract). In Vitro 28: 40A(I–1019).

Stanley-Samuelson DW (1994) Assessing the significance of prostaglandins and other eicosanoids in insect physiology. J. Insect Physiol. 40: 3–11.

Sang JH (1980) *Drosophila* cell lines. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems *in vitro* (pp. 3–12) Elsevier/North-Holland Biomedical Press, New York.

Sato T (1989) Establishment of eight cell lines from neonate larvae of tortricids (Lepidoptera) and their several characteristics including susceptibility to insect viruses. In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 2 (pp. 187–198) CRC Press Inc, Florida.

Soderhall K & Smith VJ (1983) Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, prophenoloxidase distribution. Dev. comp. Immunol. 7: 229–239.

Sohi SS (1968) *In vitro* cultivation of *Choristoneura fumiferana* (Clemins) (Lepidopteran:Tortricidae) tissues. Can. J. Zool. 46: 11–13.

Sohi SS (1971) *In vitro* cultivation of haemocytes of *Malacosoma disstria* Hubner (Lepidoptera: Lasiocampidae). Can. J. Zool. 49: 1355–1358.

Sohi SS (1973) *In vitro* culture of larval tissues of *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). In: J. Rehacek J,Blaskovic D & Hink WF (eds), Proceedings Third International Colloquim on Invertebrate Tissue Culture (pp. 75–89) Publishing House of the Slovak Academy of Sciences, Bratislava.

Sohi SS (1980) The effect of pH and osmotic pressure on the growth and survival of three Lepidopteran cell lines. In: Kurstak E, Maramorosch K & Dubendorfer A (eds) Invertebrate Systems in vitro (pp. 35–43) Elsevier/North-Holland Biomedical Press, New York.

Steel JE (1993) Serotonin: a putative regulator of glycogen metabolism in the CNS of the cockroach, *Periplaneta americana.* In: Borkovec AB & Loeb MJ (eds) Insect Neurochemistry and Neurophysiology (pp. 141–144) CRC Press, Ann Arbor.

Stipanovic RD, Elissalde MH, Altman DW & Norman JO (1990) Cell culture bioassay to evaluate allelochemical toxicity to *Heliothis virescens* (Lepidoptera: Noctuidae). J. Econ. Entomol. 83: 737–741.

Takagi M, Okumura H, Okada T, Kobayashi N, Kiyota T & Ueda K (1994) An oxygen supply strategy for the large-scale production of tissue plasminogen activator by microcarrier cell culture. J. Ferment. Bioeng. 77: 301–306

Ui K, Ueda R & Miyake T (1989)*In vitro* culture of cells from dissociated imaginal disks of *Drosophila melanogaster.* In: Mitsuhashi J (ed) Invertebrate Cell System Applications. Vol. 2 (pp. 213–221) CRC Press Inc, Florida.

Vaughn JL, Goodwin RH, Tompkins J, & McCawley P (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro 13: 213–217.

Vaughn JL (1985) Insect tissue culture: techniques and development. Techniques in the Life Sciences: Setting Up and Maintenance of Tissue and Cell Cultures C108: 1–35.

Wang M-Y & Bentley WE (1994) Continuous insect cell (Sf–9) culture with aeration through sparging. Appli. Microbiol. Biotechnol. 41: 317–323.

Ward GB, Kelly TJ, Woods CW & Marks EP (1987) Ecdysteroid production by a continuous insect cell line. Arch. Insect Biochem. Physiol. 5: 91–98.

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Special Issue

Transgenic insect cells: mosquito cell mutants and the dihydrofolate reductase gene

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Introduction

Despite the number and diversity of cell lines established from various insect species, the powerful tool of DNA-mediated gene transfer has been adapted to relatively few lines, mostly from flies *(diptera)* and moths *(lepidoptera).* Among the flies, transformation of *Drosophila melanogaster* using the transposable element P allows a direct interface with a well-developed classical genetic infrastructure, and transfection of cell lines (Walker, 1989) has thus received little attention, relative to transformed organisms. In contrast, with mosquitoes, technologies for organism level transformation are limited and inefficient (Fallon, 1991). Here, transformation efforts with cultured cells have strengthened the anticipation that transgenic stategies may eventually be implemented to disrupt disease transmission by vector species. Finally, in the lepidoptera, transformation technologies have focussed largely on cells that support replication of baculovirus expression vectors, which have been engineered for large scale production of recombinant proteins. Because lepidopteran cells carry out posttranslational modifications such as glycosylation and secretion, the well-developed baculovirus systems play a major role in expression of eukaryotic gene products of potential therapeutic value.

It is not surprising that among the non-Drosophilid insects, transformation technologies for mosquito and lepidopteran cells have evolved from different perspectives. Cells from a variety of lepidopteran species have long contributed to basic and applied aspects of baculovirus research, ranging from production of virus in large quantities for pest control (Summers & Kawanishi, 1978), to analysis of viral gene expression using the most advanced techniques of classical animal virology (Blissard & Rohrmann, 1990). Successful demonstration of abundant gene expression driven by the polyhedrin gene promoter from the baculovirus genome quickly captured the imagination of investigators interested in eukaryotic expression vectors, making cells from the noctuid moth, *Spodoptera frugiperda*, among the best known and most extensively used insect cell lines. In contrast, baculoviruses that infect mosquitoes (Federici, 1980) are virtually unknown, and even if such viruses become available in the future, mosquito cells may be considered inappropriate for production of therapeutic proteins because inadvertant contamination with RNA viruses that may be cytopathic to mammalian cells may not be apparent in mosquito cells.

For biological control of mosquitoes, little work has been done to develop viruses as control agents. Parasites other than viruses, such as *Bacillus thuringiensis* var. *israelensis* (Adang, 1991) and the microsporidian *Edhazardia aedis* (Sweeney & Becnel, 1991) have received more systematic attention. An important incentive for the establishment of mosquito cell lines relates to their potential value for investigation of arthropod-borne RNA viruses infectious to humans or domesticated animals. Work with prototypes of the arthropod-borne viruses that cause disease in humans, such as Sindbis virus, and various flaviviruses including dengue, has advanced considerably through the

examination of virus-host-cell interactions in mosquito cell lines and their somatic cell variants (Sarver & Stollar, 1977; Tatem & Stollar, 1986; Lemm *et al*., 1990; Mi & Stollar, 1990; Miller & Brown, 1992; Randolph & Stollar, 1990). In fact, recombinant Sindbis virus vectors now provide an important tool for analysis of foreign gene expression in transformed mosquitoes (Carlson *et al*., 1995). Although few DNA viruses are known to replicate in mosquito cells, recent recognition of their potential value for organism-level transformation now provides a strong incentive for their molecular characterization (Afanasieve *et al*., 1994; Boublik *et al*., 1994).

In contrast to lepidopteran cells, which are characterized by large numbers of small chromosomes and an unstable karyotype (Oberlander & Miller, 1985), cells from *Drosophila* and mosquitoes contain small numbers of large chromosomes, a feature that lends itself to somatic cell genetics. Unlike cells from *Drosophila*, mosquito cells readily adapt to vertebrate tissue culture media (Sarver & Stollar, 1977), which generally have fewer undefined components than standard media for insect cells, such as the formulation of Mitsuhashi & Maramorosch (1964) that was originally used to establish some mosquito cell lines (Singh, 1967), or the D22 medium used with some *Drosophila* lines (Bourouis & Jarry, 1983). Because undefined components in culture media compromise the generation and characterization of somatic cell mutants, it is not surprising that adaptation of selective strategies analogous to those used with mammalian cells has been most straightforward with mosquito cells. In this review, adaptations of these technologies to mosquito cells, and extension of these efforts to develop vectors and procedures for DNA-mediated gene transfer are summarized and discussed.

Somatic cell genetics

In the history of mammalian cell culture, the development of selective media based on nucleoside metabolism is often regarded as the key innovation that paved the way for somatic cell genetics, cell fusion and ultimately the modern gene transfer technologies used to investigate eukaryotic gene expression. Particularly prominent was HAT selective medium, in which hypoxanthine (H), aminopterin (A), and thymidine (T) were used in some of the earliest efforts to identify transfected cells that differed from wild type in nucleoside metabolism (Szybalska & Szybalski, 1962). More recently, mammalian cell lines deficient in enzymes essential for salvage of exogenous purine (hypoxanthine phosphoribosyl transferase) or pyrimidine (thymidine kinase) precursors have been essential in early efforts to establish efficient transfection procedures by complementing known cellular mutations with appropriate cloned genes. In particular, the early efforts to transfer viral thymidine kinase genes into mouse thymidine kinase deficient (Ltk^-) cells (Wigler *et al.,* 1977) paved the way for elegant suicide selection technologies (Ezzedine *et al*., 1991) that take advantage of differences in the sensitivity of viral versus cellular TK enzymes to antiherpetic drugs.

Why did nucleoside metabolism play such a large role in the development of somatic cell genetics? Purine and pyrimidine nucleosides are stable compounds, their concentrations in serum are low, and they are not generated by breakdown of other macromolecules in serum. Mammalian cells produce purine and pyrimidine precursors *de novo*; thus, these compounds are not required in culture medium for survival of wild type cells. However, salvage pathways for purine and pyrimidine nucleosides allow survival of mutants when appropriate supplements are added to the culture medium (Figure 1). Moreover, in mammalian cells, the frequency of mutations in nucleoside metabolism is sufficiently high for recovery of both forward and reverse mutations. Finally, interest in nucleoside metabolism in cultured cells was complemented by the use of analogs in cancer chemotherapy, and the observation that drug-resistant variants arose from tumor cell populations (Morrow, 1982).

Mosquito cell mutants

The systematic application of somatic cell genetics to a cell line from the mosquito, *Aedes albopictus*, was pioneered by the efforts of Mento & Stollar (1978), who demonstrated the use of ethylmethane sulfonate to mutagenize mosquito cells, and recovered clones resistant to 5-bromodeoxyuridine, ouabain, and *a*-amanitin. Since that time, several somatic cell mutants have been derived from mosquito cells (for a review see Fallon & Stollar, 1987) and biochemical approaches have been developed to facilitate molecular studies with these mutants. In particular, substantial progress in the analysis of nucleoside metabolism has shown that these mosquito cells differ in important ways from vertebrate cells. For example, although mosquito cells salvage thymidine from the medium (Sherwood & Stollar, 1982), they are unable to incorporate radi-

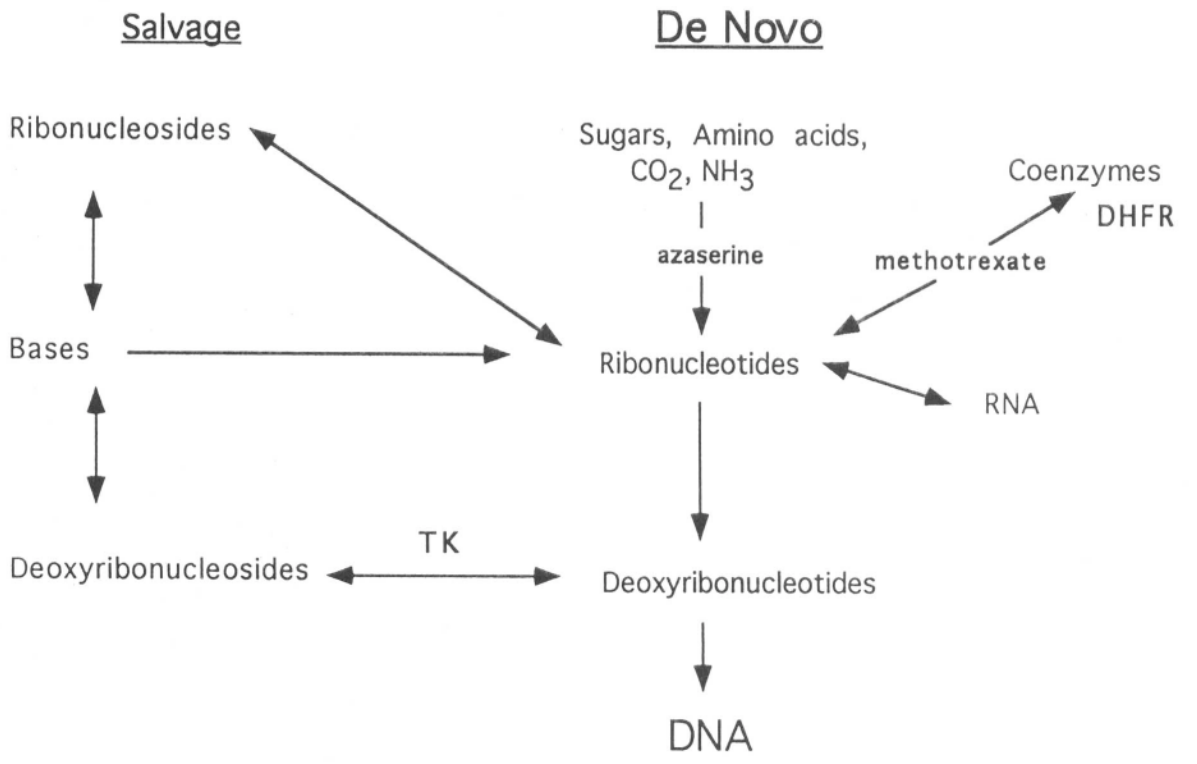

Figure 1. Simplified representation of salvage and de novo pathways of purine and pyrimidine biosynthesis. TK, thymidine kinase; DHFR, dihydrofolate reductase; azaserine, purine inhibitor; methotrexate, folate inhibitor.

olabelled hypoxanthine into nucleic acid (Malinoski & Stollar, 1981), precluding use of HAT selective medium. Efforts to express a bacterial xanthine guanine phosphoribosyl transferase gene in mosquito cells led to the observation that untransfected cells phosphoribosylate xanthine (Swerdel & Fallon, 1987). Finally, when tk^-, ouabain-resistant cells (Oua 5-6 cells) were used in our own early efforts to develop gene transfer protocols using a cloned viral tk gene, recovery of tk^+ transformants in a selective medium analogous to HAT (containing methotrexate and the four ribo- and deoxyribonucleosides) was limited, but tk^- clones surviving at methotrexate concentrations approximately 50-fold higher than those used to select methotrexate-resistant mammalian cells were recovered (Fallon, 1984). Effective recovery of transformed mosquito cells in which the tk^- phenotype was stably rescued by expression of the tk gene from herpes simplex virus has only recently been achieved, using a selective medium in which the *de novo* pathway for nucleoside biosynthesis was doubly blocked with methotrexate and azaserine (Mazzacano & Fallon, 1995).

Among the *lepidoptera,* thymidine kinase-deficient mutants have recently been described from *Spodoptera frugiperda* (Xie *et al.*, 1991) and *Spodoptera exigua* (McCarthy & McKedy, 1990) cell lines. With *S. frugiperda* cells, mutagenesis with ethylmethane sulfonate followed by selection with 5-bromodeoxyuridine allowed recovery of clones 100–200 fold more resistant to 5-bromodeoxyuridine than the parental cells. Unlike mosquito cells, the wild type *S. frugiperda* cells were highly resistant to aminopterin (methotrexate). With these cells, selection of cells transformed with a viral tk gene was accomplished using HAT medium supplemented with the folate antagonist sulfanilamide (Xie *et al.*, 1991).

Transfection of mosquito cells: methodology

Our initial efforts to transfect tk^- mosquito cells with the cloned tk gene from herpes simplex virus were hindered by the lack of a procedure known to introduce DNA efficiently into these cells, and uncertainty as to whether the tk promoter from a virus

that typically replicates in mammalian cells would be adequately recognized by the transcriptional machinery of insect cells. These limitations were overcome when the hsp-cat 1 plasmid was developed for transfection of *Drosophila* cells (Di Nocera & Dawid, 1983). This plasmid, containing a bacterial chloramphenicol acetyltransferase gene under the control of a temperature-inducible promoter from the heat shock protein (hsp) 70 gene of *Drosophila,* facilitated establishment of a calcium phosphate-mediated gene transfer protocol for *D. melanogaster* and *D. immigrans* cells. Because the *Drosophila* hsp 70 promoter had already been shown to function in diverse heterologous systems (for references, see Gerenday *et al*., 1989), we reasoned that the promoter driving expression of the CAT gene in this construct would be effective in mosquito cells. With hsp-cat 1, we readily defined conditions for transient transfection of *Aedes albopictus* C7-10 cells using the polycation polybrene as the chemical mediator (Durbin & Fallen, 1985; Fallon, 1989). Although subsequent efforts later demonstrated that calcium phosphate can also be used effectively with these cells (Kjer & Fallon, 1991), the greater simplicity and reproducibility of the polybrene procedure leaves calcium phosphate the mediator of choice only when it becomes necessary to remove unincorporated DNA.

Although hsp-cat 1 has now been expressed in several insect cell lines, a systematic analysis of the various transfection procedures with diverse insect cells, which might yield useful predictive information, remains to be undertaken. It is of interest to note, however, that some *Drosophila* cell lines that are difficult to transfect using calcium phosphate respond more readily to polybrene (Walker, 1989), and that the commercially available lipofection is routinely used for transfection of *Aedes albopictus* C6-36 cells (Monroe *et al.,* 1992). In the course of our own studies, we showed that hsp-cat 1 can be expressed in *Spodoptera frugiperda* IPLB-SF-21 cells after polybrene-mediated transfection, provided the heat shock conditions for induction of CAT expression are adapted to the physiology of the recipient cell (Gerenday *et al*., 1989; Helgen & Fallon, 1990). Based on histochemical staining, the transfection frequency with *Spodoptera* was about 1/1000 cells-ten-fold higher than what we routinely see with *Aedes* cell (Gerenday *et al*., 1989). With *Spodoptera* cells, calcium phosphate is commonly used for transfection of baculovirus constructs, and effective selection systems have eliminated the need for extensive optimization of transfection procedures.

Methotrexate resistance, revisited

Our unanticipated recovery of methotrexate-resistant mosquito cells from early gene transfer experiments was fortuitous, because major research efforts in several laboratories had already established methotrexate resistance in mammalian cell culture as a primary model for studies on gene amplification (for reviews see Hamlin & Ma, 1990; Schimke, 1980, 1984). Methotrexate-resistant cells provided an opportunity to amplify, and easily clone, the essential housekeeping gene encoding dihydrofolate reductase (dhfr). Mammalian dhfr genes had already been cloned, and their cDNAs (or specific modifications thereof) were already being used as selectable markers to recover stably transfected cells, in which the cotransfected genes could be amplified using methotrexate (McIvor & Simsonsen, 1990; Kaufman *et al*., 1991). Ongoing efforts to clone the *Drosophila* dhfr gene from methotrexate-resistant cells (Hao *et al*., 1994) further stimulated our interest in the mosquito gene.

As with mammalian cells, methotrexate resistance in mosquito cells could occur for many different reasons. For example, mutations affecting transport of methotrexate from the culture medium into the cell, point mutations that decreased the affinity of DHFR protein for methotrexate, and an increase (amplification) in the number of dhfr genes resulting in synthesis of excess DHFR protein have all been shown to account for resistance in various mammalian clones. The possibility of gene amplification was of particular interest to us, because studies with insects in the field had already implicated amplification of esterase genes as the genetic basis for organophosphate resistance in aphids and mosquitoes (Devonshire & Field, 1991). In fact, in mosquitoes, an amplification event that apparently took place once has now spread throughout the world (Raymond *et al*., 1991), suggesting that in at least some cases, interference with the genetic events that lead to gene amplification may provide a useful target in transgenic insect applications. In this respect, a cell culture system in which gene amplification could be manipulated *in vitro* offers an insight into molecular processes that underlie an important problem in applied entomology.

Cloning a mosquito dhfr gene

From cells that showed an initial 6.5-fold increase in resistance to methotrexate relative to wild type cells

(Fallon, 1984), we used step-wise selection to gradually obtain cells in which resistance was 3000-fold increased (Shotkoski & Fallon, 1990). These cells overproduced a protein corresponding in size and N-terminal amino acid sequence to vertebrate DHFR proteins, with 45% homology over the first 20 amino acids (Johnston & Fallon, 1987). In the most highly resistant Mtx-5011-256 cells, DHFR constituted an estimated 30% of total soluble protein (Shotkoski & Fallon, 1990). Overproduction of DHFR protein, as well as overproduction of dhfr mRNA and the hybridization of cDNA synthesized from this mRNA to a unique 8.5 kb EcoRI fragment in genomic DNA from resistant cells were consistent with amplification of the mosquito dhfr gene. The 8.5 kb EcoRI fragment was found to contain the complete dhfr gene, which in Mtx-5011-256 cells was present at approximately 1200 copies per nucleus (Shotkoski & Fallon, 1991). Based on pulsed-field gel electrophoresis and Southern blotting, we have shown that the dhfr locus lies within an amplicon that extends at least 140 kb, and possibly as long as 233 kb (Shotkoski & Fallon, 1993a).

Structure of the mosquito DHFR gene

Dihydrofolate reductase genes from mouse, hamster, and humans are complex: they extend over approximately 30 kb of DNA and are interrupted by 5 introns, one of which measures as much as 16 kb (Yang *et al.*, 1984). In contrast, hybridization of the mosquito dhfr probe to a single 8.5 kb fragment unique to EcoRI-digested genomic DNA from resistant cells suggested that the entire coding sequence was contained within this single 8.5 kb fragment. Subsequent analysis localized the dhfr gene within a 1.8 kb AccI subfragment (Shotkoski & Fallon, 1991). The gene has been sequenced, and shown to contain a single 56 bp intron that interrupts a phylogenetically conserved Arg codon at amino acid position 27. The absence of additional introns suggested that the single conserved intron plays an essential regulatory role. In support of this possibility, we note that mouse dhfr genes contain sequences with orientation- and position-independent enhancer activity that extend from the end of the first exon into the first intron (Farnham & Means, 1990; Schmidt *et al.*, 1990). Moreover, in *Drosophila melanogaster* the dhfr gene (which was not amplified in response to methotrexate) has now been cloned and sequenced. Like the *Aedes albopictus* gene, the *Drosophila* gene contains a single small (50 bp) intron, corresponding to the mosquito intron, and to the first intron of dhfr genes from vertebrates (Hao *et al.*, 1994).

Stable transfection

Because the technology for transfection of insects at the level of the organism awaits the characterization of appropriate transposable elements, a long term goal of our research is to use insect cell culture to explore methods than can later be applied to transgenic insects. The compact structure of the *A. albopictus* dhfr gene simplified its use as a dominant selectable marker in transfected cells, because we were able to use the cloned gene itself, under the regulation of its endogenous promoter, rather than a derived cDNA copy (Crouse *et al.*, 1983). We first identified the transcription initiation sites for the mosquito dhfr gene using Northern analysis, primer extention, and RNAase protection experiments, thus ensuring that the available genomic DNA clone contained sufficient information for regulation of gene expression (Park & Fallon, 1993). The upstream flanking DNA contained TATA and CAAT boxes, but the 48 bp GC repeats typical of mammalian dhfr genes were absent. In both sensitive and methotrexate-resistant cells, transcription of the dhfr gene initiated at the same set of alternative sites, which generated minor heterogeneity at the 5'-end of dhfr transcripts (Park & Fallon, 1993). Based on this information, the *Aedes* dhfr gene and ~500 bp of endogenous upstream sequence was cloned into the Bluescript II plasmid, and also into the hsp-cat-1 plasmid of Di Nocera & Dawid (1983) to generate a composite plasmid (pDHFR:cat-l) containing both the dhfr gene with endogenous regulatory sequences and the bacterial chloramphenicol acetyltransferase (CAT) gene, under the control of a *Drosophila melanogaster* hsp 70 promoter. This construct, as well as a construct containing only the mosquito dhfr gene and its upstream sequences (pFSDHFR9), have been introduced into cultured cells by polybrene-mediated transfection. After a 48-h post-transfection recovery period, stable transformants were selected after 2 weeks in the presence of 1 μM methotrexate and screened for CAT activity after heat shock induction. All clones examined were positive for CAT activity, i.e., contained transfected DNA, which was estimated at 100-500 copies/nucleus (Shotkoski & Fallon, 1993b). When hsp-cat 1 and pFSDHFR9 were introduced simultaneously into cells by co-transfection, stable transformants acquired both resistance to methotrexate and

temperature-inducible CAT activity. Thus, the dhfr gene can be used as a dominant selectable marker in a composite plasmid or in technically simpler cotransfection procedures, which do not require construction of composite vectors. This phenomenon demonstrates that as with mammalian cells, mosquito cells that are physiologically "competent" to take up DNA incorporate multiple DNA species.

Why is the wild type mosquito dhfr gene an effective selectable marker?

In vertebrate cells, dhfr transfection vectors similar to those developed for mosquito cells function well only when introduced into dhfr-deficient recipient cells (Kaufman & Sharp, 1982; Crouse *et al.*, 1983). In contrast, our constructs functioned effectively as selectable markers in wild-type recipient cells, allowing use of the plasmids as selectable markers for cotransfection in any recipient mosquito cell line, without the laborious effort of developing dhfr-deficient derivatives. Recent studies implicate both methotrexate-affinity and catalytic activity (Hussain *et al.*, 1992) as factors that allow mosquito DHFR to function as a dominant selectable marker in wild-type cells. Apparently, both *Aedes* and *Drosophila melanogaster* DHFR enzymes (Rancourt & Walker, 1990) have in common a higher k_d for methotrexate than non-insect DHFRs. This reduced affinity for methotrexate contributes to its versatility as a dominant selectable marker, similar to that of "mutant" dhfr constructs containing specific amino acid replacements in cloned vertebrate dhfr cDNAs (Simonsen & Levinson, 1983; Hussain *et al.*, 1992; McIvor & Simonsen, 1990).

We have used the dhfr gene as a dominant selectable marker to introduce into mosquito cells a second, "antisense dhfr" construct whose expression was regulated by the *Drosophila* hsp70 promoter. Cell survival (as determined by growth kinetics and plating efficiency) was disrupted under moderate (34 °C) heat shock conditions, where low levels of antisense transcripts were induced (Gerenday *et al.*, 1989; Shotkoski & Fallen, 1994). The results support the potential value of the dhfr gene for developing genetic approaches to interfere with manipulatable functions in insects-growth, development, reproduction-using DNA constructs that can be tested directly in cell culture, and adapted to pest species as the appropriate transfer technologies for embryos become available.

Future studies

We anticipate that the mosquito dhfr gene will continue to play a role in our ongoing efforts to improve transgenic technologies for *A. albopictus* cells. Three areas of research are of particularly high priority, and are supported by existing work with vertebrate systems which provide an important framework for extending these analyses to mosquitoes. These efforts include characterization of the structure of the dhfr amplicon, identification of transcription units and repeated sequence elements within the amplicon, and identifying a replication origin that, once characterized, might be incorporated into gene transfer vectors.

Although the generation of multiple copies of the dhfr gene is loosely termed "dhfr gene amplification", the total size of the amplified unit is significantly larger than the dhfr gene itself. Thus, substantial amounts of flanking DNA are coamplified with the gene. Mouse and hamster dhfr amplicons that have been investigated in detail range from 250 kb to over 10,000 kb in size (reviewed by Devonshire & Field, 1991). In the typical situation, however, an initially large amplicon stabilizes at the smaller size during extended time in culture. Given that the mammalian dhfr coding sequence spans approximately 30 kb, a 250 kb amplicon is roughly 10 times larger than the gene itself. With the small size (0.6 kb) of the mosquito dhfr gene and its single 56 bp intron, 1 250 kb amplicon would contain roughly 400-fold more DNA than is needed to encode the selectable gene. Current studies suggest that there may be alternative forms of the mosquito dhfr amplicon (Wang & Fallon, unpublished), and efforts to learn how the amplicon structure in resistant cells compares to the arrangement of non-amplified DNA in wild type cells may ultimately provide a characterized locus for analyzing targeted gene replacement in insect cells.

Repeated sequence elements have been shown to occur in vertebrate dhfr amplicons (Anachkova & Hamlin, 1989). It will be of interest to learn whether any such elements occur in the mosquito dhfr amplicon, if the same elements occur elsewhere in the genome and if so, whether they occur in wild type, sensitive cells. In CHO cells, at least three additional transcription units have been indentified within a 273 kb dhfr amplicon (Foreman & Hamlin, 1989). In future studies we plan to explore whether other transcription units lie within the mosquito dhfr amplicon.

Finally, our interest in defining a replication origin within the dhfr amplicon relates to its potential value as a tool for further extension of gene trans-

fer technologies. In simple organisms such as bacteria, viruses and some lower eukaryotes, functional and genetic mapping of DNA replication origins identify the same locus, which measures on the order of 50 to 1000 bp (dePamphilis, 1993a). These origins consist of well-defined sequences that interact with specific proteins (for reviews, see dePamphilis, 1993a,b). Components of the replication origin include a recognition sequence, a DNA-unwinding element, and binding sites for specific transcription factors. Origins of replication in complex organisms are less well understood, but the available model systems predict that 1) unique cis-acting sequences determine where replication begins, and 2) chromatin structure and nuclear organization distinguish preferred initiation sites from other potential sites. Origins of DNA replication have been likened to transcriptional promoters, in that both elements contain modular components that engage in ordered interactions with multiple proteins. Once characterized, the molecular properties of replication origins and the proteins with which they interact will provide an important tool for understanding the details of insect growth and development, and also serve as potential genetic targets in transgenic insects.

The elegant studies of Hamlin and associates with methotrexate-resistent Chinese hamster ovary cells (Heintz *et al*., 1983; Heintz & Hamlin, 1982) provide a model for identifying which restriction fragments in the mosquito dhfr amplicon contain chromosomal origins of replication. Briefly, their analysis took advantage of the fact that the dhfr locus replicates early in the S (DNA synthesis) phase of the cell cycle (Milbrandt *et al*., 1981). Based on a known amplicon map, early-labeled fragments (e.g., containing origins) were identified by labeling synchronized cells with [^{14}C]thymidine upon reentry to the S phase of the cycle. Cells were then treated with inhibitors of DNA synthesis to confirm the locations of replication origins to these "early labelled fragments" (Burhans *et al*., 1986a,b; Leu & Hamlin, 1989). Although 3 different origins have been identified in the CHOC 400 cells (reviewed by dePamphilis, 1993a,b), most of the research to date has focused on ori-β, which maps between two matrix attachment regions-elements which have also been associated with origins in model systems. Ori-β is also flanked by Alu repeats, and contains a segment of bent DNA with sequence similarity to yeast ARS (autonomously replicating sequence) consensus elements. Although it will take considerable time and effort to document these features of replication origins in this mosquito cell culture system, these insights are likely to contribute to the construction of improved transfection vectors.

Mysteries solved; mysteries remain

During the past decade, we have made considerable progress in the use of mosquito somatic cell genetics to advance gene transfer technologies. In comparison with the parallel efforts undertaken with *Drosophila* cells, and in the absence of controlled, systematic investigations, we can only speculate as to why it was relatively easy to obtain amplified dhfr genes from mosquito cells, or conversely, why methotrexate-resistant *Drosphila* cells failed to amplify the dhfr gene. We note that the methotrexate-resistant mosquito cells arose fortuitously from parental Oua 5-6 cells that were already tk$^-$, and that this k$^-$ phenotype has remained stable, despite many years of maintenance in media lacking 5-bromodeoxyuridine(Wang & Fallen, unpublished). Because Oua 5–6 cells were inherently unable to use thymidine by the salvage pathway, and our early efforts at gene transfer were marginally efficient at best (see Fallon, 1984; Kjer & Fallon, 1991), we suggest that methotrexate selection was a predominant factor in the recovery of mutants that had undergone dhfr gene amplification. Are wild type cells, which have a functional salvage pathway, less likely than tk$^-$ cells to generate variants in which amplification of an essential gene in the *de novo* pathway has occurred? Consistent with this possibility is our failure thus far to recover methotrexate-resistant, nontransformed clones from wild type mosquito cells transfected with constructs containing the dhfr gene and subjected to methotrexate selection. Thus, at the μM concentrations used in these studies, methotrexate reliably discriminates among wild type cells and cells that acquire dhfr genes by transfection, suggesting that the frequency of spontaneous amplification of dhfr genes in wild type cells is much lower than the transfection frequency. In contrast to this situation with mosquito cells, tk$^-$ cells have never been reported from *Drosophila*, and attempts to amplify the dhfr locus were based on methotrex-- ate selection from wild type cells. It will thus be of interest to learn whether tk$^-$ parental cells would enhance recovery of methotrexate-resistant *Drosophila* cells that contain amplified dhfr genes, and to understand in greater detail how the phenotype of recipient cells influences the relative outcome of gene transfer events.

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References

Adang MJ (1991) Bacillus thuringiensis insecticidal crystal proteins: Gene structure, action and utilization. In: Maramorosch K (ed.) Biotechnology and biological control of pests and vectors. (pp 3-25) CRC Press, Boca Raton, FL.

Afanasiev BN, Kozlov YV, Carlson JO & Beaty BJ (1994) Densovirus of *Aedes aegypti* as an expression vector in mosquito cells. Expt. Parasitol. 79: 322–332.

Anachkova B & Hamlin JL (1989) Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. Mol. Cell. Biol. 9: 532–540.

Blissard GW & Rohrmann GF (1990) Baculovirus diversity and molecular biology. Annu. Rev. Entomol. 35: 127–155.

Boublik Y, Jousset F-X & Bergoin M (1994) Complete nucleotide sequence and genomic organization of the *Aedes albopictus* parvovirus (AaPV) pathogenic for *Aedes aegypti* larvae. Virology 200: 752–763.

Bourouis M & Jarry B (1983) Vectors containing a prokaryotic dihydrofolate reductase gene transform *Drosophila* cells to methotrexate-resistance. EMBOJ. 2: 1099–1104.

Burhans WC, Selegue JE & Heintz NH (1986a) Isolation of the origin of replication associated with the amplified Chinese hamster dihydrofolate reductase domain. Proc. Natl. Acad. Sci. USA 83: 7790–7794.

Burhans WC, Selegue JE & Heintz NH (1986b) Replication intermediates formed during initiation of DNA synthesis in methotrexate-resistant CHOC 400 cells are enriched for sequences derived from a specific, amplified restriction fragment. Biochemistry 25: 441–449.

Carlson J, Olson K, Higgs S & Beaty B (1995) Molecular genetic manipulation of mosquito vectors. Annu. Rev. Entomol. 40: 359–388.

Crouse GF, McEwan RN & Pearson ML (1983) Expression and amplification of engineered mouse dihydrofolate reductase minigenes. Mol. Cell. Biol. 3: 257–266.

dePamphilis ML (1993a) Origins of DNA replication in metazoan chromosomes. J. Biol. Chem. 268: 1–4.

dePamphilis ML (1993b) Eukaryotic DNA replication: Anatomy of an origin. Annu. Rev. Biochem. 62: 29–63.

Devonshire AL & Field LM (1991) Gene amplification and insecticide resistance. Annu. Rev. Entomol. 36: 1–23.

Di Nocera PP & Dawid IB (1983) Transient expression of genes introduced into cultured cells of *Drosophila.* Proc. Natl. Acad. Sci. USA 80: 7095–7098.

Durbin JE & Fallon AM (1985) Transient expression of the chloramphenicol acetyltransferase gene in cultured mosquito cells. Gene 36: 173–178.

Ezzeddine ZD, Martuza RL, Platika D, Short MP, Malik A, Choi B & Breakefield XO (1991). Selective killing of glioma cells in culture and *in vivo* by retrovirus transfer of the Herpes simplex virus thymidine kinase gene. New Biol. 3: 608–614.

Fallon AM (1984) Methotrexate resistance in cultured mosquito cells. Insect Biochem. 14: 697–704.

Fallon AM (1989) Optimization of gene transfer in cultured insect cells. J. Tissue Culture Methods 12: 1–6.

Fallon AM (1991) DNA-mediated gene transfer: applications to mosquitoes. Nature 352: 828-829.

Fallon AM & Stollar V (1987) The biochemistry and genetics of mosquito cells in culture. In: Maramorosch K (ed.). Advances in Cell Culture vol. 5 (pp 97–137) Academic Press, NY.

Farnham PJ & Means AL (1990) Sequences downstream of the transcription initiation site modulate the activity of the murine dihydrofolate reductase promoter. Mol. Cell. Biol. 10: 1390–1398.

Federici BA (1980) Mosquito baculovirus: sequence of morphogenesis and ultrastructure of the virion. Virology 100: 1–9.

Foreman PK & Hamlin JL (1989) Identification and characterization of a gene that is coamplified with dihydrofolate reductase in a methotrexate-resistant CHO cell line. Mol. Cell. Biol. 9: 1137–1147.

Gerenday A, Park Y-J, Lan Q & Fallon AM (1989) Expression of a heat-inducible gene in transfected mosquito cells. Insect Biochem. 19: 679–686.

Hamlin JL & Ma C (1990) The mammalian dihydrofolate reductase locus. Biochim. Biophys. Acta 1087: 107–125.

Hao H, Tyshenko MG & Walker VK (1994) Dihydrofolate reductase of *Drosophila:* cloning and expression of a gene with a rare transcript. J. Biol. Chem. 269: 15179–15185.

Heintz NH & Hamlin JL. (1982) An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. USA 79: 4083–4087.

Heintz NH, Milbrandt JD, Greisen KS & Hamlin JL (1983) Cloning of the initiation region of a mammalian chromosomal replicon. Nature 302: 439–441.

Helgen JC & Fallon AM (1990) Polybrene-mediated transfection of cultured lepidopteran cells: Induction of a *Drosophila* heat shock promoter. In Vitro Cell. Dev. Biol. 26: 731–736.

Huberman JA, Spotila LD, Nawotka KA, E1-Assouli, SM & Davis LR (1987). The *in vivo* replication origin of the yeast 2 μm plasmid. Cell 51: 473–481.

Hussain A, Lewis D, Myounghee Y & Melera PW (1992) Construction of a dominant selectable marker using a novel dihydrofolate reductase. Gene 112: 179–188.

Johnston AM & Fallon AM (1987) N-terminal amino acid sequence analysis of dihydrofolate reductase from methotrexate-resistant mosquito cells. Archs. Insect Biochem. Physiol. 5: 57–69.

Kaufman KD, Foti D, Seto P & Rapoport B (1991) Overexpression of an immunologically-intact, secreted form of human thyroid peroxidase in eukaryotic cells. Molec. Cell. Endocrinol. 78: 107–114.

Kaufman RJ & Sharp PA (1982) Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary DNA gene. J. Molec. Biol. 159: 601–621.

Kjer KM & Fallon AM (1991) Efficient transfection of mosquito cells is influenced by the temperature at which DNA-calcium phosphate coprecipitates are prepared. Archs. Insect Biochem. Physiol 16: 189–200.

Lemm JA, Durbin RK, Stollar V & Rice CM (1990) Mutations which alter the level or structure of nsP4 can affect the efficiency

of Sindbis virus replication in a host-dependent manner. J. Virol. 64: 3001–3011.

Leu TH & Hamlin JL (1989) High resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. Molec. Cell. Biol. 9:523–531.

Malinoski F & Stollar V (1981) Inhibition of Sindbis virus replication by ribavirin: Influence of cultural conditions and of the host cell phenotype. Antiviral Res. 1: 287–299.

Mazzacano CA & Fallon AM (1995). A model for suicide selection in mosquitoes: Expression of a viral thymidine kinase gene in stably transformed cells. Insect Molec. Biol. 4: 125–134.

McCarthy WJ & McKedy D (1990) Replication of Autographa californica nuclear polyhedrosis virus in a thymidine kinase deficient *Spodoptera exigua* cell line (SE-UCR-1A) In vitro Cell. Dev. Biol. 26: 824–828.

McIvor RS & Simonsen CC (1990) Isolation and characterization of a variant dihydrofolate reductase cDNA from methotrexate-resistant murine L5178Y cells. Nucleic Acids Res. 18: 7025–7032.

Mento SJ & Stollar V (1978) Isolation and partial characterization of drug-resistant *Aedes albopictus* cells. Somat. Cell Genet. 4: 179–191.

Mi S & Stollar V (1990) Both amino acid changes in nsPl in Sindbis virus $_{LM21}$ contribute to and are required for efficient expression of the mutant phenotype. Virology 178: 429–434.

Milbrandt JD, Heintz NH, White WC, Rothman SM & Hamlin JL (1981) Methotrexate-resistant Chinese hamster ovary cells have amplified a 135kb pair region that includes the dihydrofolate reductase gene. PNAS 78: 6043–6047.

Miller ML & Brown DT (1992) Morphogenesis of *Sindbis* virus in three subclones of *Aedes albopictus* (mosquito) cells. J. Virol. 66: 4180–4190.

Mitsuhashi J & Maramorosch K (1964) Leafhopper tissue culture: Embryonic, nymphal and imaginal tissues from aseptic insects. Contrib. Boyce Thompson Inst. 22: 435–360.

Monroe TJ, Muhlmann-Diaz MC, Kovach MJ, Carlson JO, Bedford JS & Beaty BJ (1992) Stable tranformation of a mosquito cell line results in extraordinarily high copy numbers of the plasmid, Proc. Natl. Acad. Sci. USA 89: 5725–5729.

Morrow J (1982) Selection of purine and pyrimidine nucleoside analog resistance in mammalian cells. In: Shay JW (ed.) Techniques in Somatic Cell genetics (pp 1–9) Plenum Press, New York.

Oberlander H & Miller S (1987) Lepidopteran cell lines: Tools for research in physiology, development and genetics. In: Maramorosch K (ed.) Advances in Cell Culture vol. 5 (pp 187–207) Academic Press, NY.

Park Y-J & Fallon AM (1993) Transcripts from a mosquito dihydrofolate reductase gene: evidence for heterogeneity at the 5' end. Insect Biochem. Molec. Biol. 23: 255–262.

Rancourt SL & Walker VK (1990) Kinetic characterization of dihydrofolate reductase from *Drosophila melanogaster.* Biochem. Cell Biol. 68: 1075–1082.

Randolph, VB & Stollar V (1990). Low pH-induced fusion in flavivirus-infected *Aedes albopictus* cell cultures. J. Gen. Virol. 71: 1845–1850.

Raymond M, Callaghan A, Fort P & Pasteur N (1991) Worldwide migration of amplified insecticide resistance genes in mosquitoes. Nature 350: 151–153.

Sarver N & Stollar V (1977) *Sindbis* virus-induced cytopathic effect in clones of *Aedes albopictus* (Singh) cells. Virology 80: 390–400.

Schimke RT (1980) Gene amplification and drug resistance. Scientific American 243: 60–69.

Schimke RT (1984) Gene amplification in cultured animal cells. Cell 37: 705–713.

Schmidt EE, Owen RA & Merrill GF (1990) An intragenic region downstream from the dihydrofolate reductase promoter is required for replication-dependent expression. J. Biol. Chem. 265: 17397–17400.

Sherwood A & Stollar V (1982) *Aedes albopictus* cells resistant to adenosine because of a defect in nucleoside transport. Somatic Cell Genet. 8: 575–585.

Shotkoski FA & Fallon AM (1990) Genetic changes in methotrexate-resistant mosquito cells. Archs. Insect Biochem. Physiol. 15: 79–92.

Shotkoski FA & Fallon AM (1991) An amplified insect dihydrofolate reductase gene contains a single intron. Eur. J. Biochem. 201: 157–160.

Shotkoski FA & Fallon AM (1993a) An amplified mosquito dihydrofolate reductase gene: amplicon size and chromosomal distribution. Insect Molec. Biol. 2: 155–161.

Shotkoski FA & Fallon AM (1993b) The mosquito dihydrofolate reductase gene functions as a dominant selectable marker in transfected cells. Insect Biochem. Molec. Biol. 23: 883–893.

Shotkoski FA & Fallon AM (1994) Expression of an antisense dihydrofolate reductase transcript in transfected mosquito cells: Effects on growth and plating efficiency. Am. J. trop. Med. Hygiene. 50: 433–439.

Simonsen CC & Levinson AD (1983) Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA 80: 2495–2499.

Singh KRP (1967). Cell cultures derived from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). Curr. Sci. 36: 506–508.

Summers MD & Kawanishi CY, eds (1978) Viral Pesticides: present knowledge and potential effects on public and environmental health: Symposium Proceedings Health Effects Research Laboratory, United States Environmental Protection Agency, Research Triangle Park, NC, 312 pp.

Sweeney AW and Becnel JJ (1991) Potential of microsporidia for the biological control of mosquitoes. Parasitol. Today 7: 217–220.

Swerdel MR & Fallon AM (1987) Phosphoribosylation of xanthine by extracts from insect cells. Insect Biochem 17: 1181–1186.

Szybalska EH & Szybalski W (1962) Genetics of human cell lines, IV. DNA-mediated heritable transformation of a biochemical trait. Proc Natl. Acad. Sci. USA 48: 2026–2034.

Tatem J & Stollar V (1986) Dominance of the CPE(+) phenotype in hybrid *Aedes albopictus* cells infected with *Sindbis* virus. Virus Research 5: 121–130.

Walker VK (1989) Gene transfer in insects. In: Maramorosch K & Sato G (eds) Advances in Cell Culture vol. 7 (pp 87–124) Academic Press, New York.

Wigler M, Silverstein S, Lee L-S, Pellicer A, Cheng Y & Axel R (1977). Transfer of a Herpes virus thymidine kinase gene to cultured mouse cells. Cell 11: 223–232.

Xie W, He D, Wang X, Long Q & Pu, Z (1991). Lepidopteran cell variants resistant to 5-bromodeoxyuridine and their use for transfection of the HSV-TK gene. J. Cell. Sci. 100: 243–247.

Yang JK, Master JN & Attardi G (1984) Human dihydrofolate reductase gene organization. J. Mol. Biol. 176: 169–187.

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Insect cell physiology

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Key words: insect cells, metabolism, energetics, amino acids, fluxes, oxygen uptake

Introduction

Viruses from the genus Baculovirus, are attractive as pesticides and as vectors for producing foreign proteins in insect cells. because insect cells are relatively easy to cultivate, faithfully perform some of the post-translational modifications, allow for complex formation to take place between the two proteins co-expressed in cultures infected with two hybrid viruses, and often can produce large quantities of proteins, baculovirus technology has found its way to a number of undustrial applications for expression of a variety of proteins (see review Murhammer, 1991; O'Reilly *et al.*, 1992). In addition to proteins, production of baculovirus itself as insecticide represents a major potential for use of insect cell cultures (Wood, 1995). The final product yield whether a protein or the virus, is influenced by several factors such as the cell line (eg. Lynn & Hink, 1980; McIntosh & Grasela, 1984; Hink *et al.*, 1991; Wickham *et al.*, 1992) and virus type (Fraser, 1989), passage number of virus (Wickham *et al.*, 1991), medium composition (eg. Cho *et al.*, 1989; Hink *et al.*, 1991), dissolved oxygen concentration (eg. Scott *et al.*, 1992; Wang *et al.*, 1993), time and the multiplicity of infection (MOI) (Licari & Bailey, 1991; Bedard *et al.*, 1994) nature of protein (Hink *et al.*, 1991), cell density (Wickham *et al.*, 1992) and stage of growth and metabolism (eg. Caron *et al.*, 1990; Lindsay *et al.*, 1992; Reuveny *et al.*, 1993).

Despite significant advance in the genetics of Baculovirus/insect cell expression system, our understanding of cellular physiology during pre- and post infection is relatively limited. An expanded understanding of metabolic features of insect cells will prove extremely useful to the bioprocess and biochemical engineers who work towards improving insect cell culture productivity. Several recent articles have addressed consumption of carbohydrate and amino acid and formation of the metabolic by-products in insect cell cultures. Those studies, reviewed first in this article, have significantly enhanced the metabolic knowledge of insect cells. Substantially more insights, however, may be obtained by a regirous analysis of primary metabolic pathways. Our recent work (Ferrance *et al.*, 1993), has demonstrated the feasibility of metabolic flow analysis for insect cell growth in a serum-free medium. This manusscript also provides a summary of metabolic pathway analysis and highlights the assumptions, describes the lessons learned, and ourlines the future work in a more critical fashion than is available in ferrance *et al.* (1993). Finally, future research directions critical for attaining detailed metabolic insights are outlined in the last section of this manuscript.

Carbohydrated and amino acid metabolism

Carbohydrate metabolism

The range and efficiency of carbohydrate utilization differ for different insect cell lines. Stockdale & Gardiner (1976) studied the utilization of 21 sugars for the growth of Trichoplusia ni (TN 368) and found that only five supported cell growth: glucose, fructose, mannose, maltose and trehalose. Glucose and trehalose were consumed at a somewhat higher rate than fructose. reuveny *et al.* (1992) also reported that a higher cell density of Sf9 cells and recombinant protein (β-galactosidase)

production was achieved in glucose containing media than in media which contained either fructose or maltose. In the presence of both glucose and fructose in the media, fructose utilization begins upon depletion of glucose at a rate comparabler to glucose (Bedard *et al*., 1993). This behavior may indicate the presence of a phenomenon similar to the catabolite repression commanded by glucose as observed for some other cells (Mathews & Holde, 1990). Maltose consumption, in media which contain both glucose and maltose, usually occurs during early growth phase as is evident from the initial rise in glucose level and the decline in maltose concentration (Ferrance *et al*., 1993; Bedard *et al*., 1993). This observation could indicate the presence of an anzyme, on the cell surface or secreted in the media, capable of breaking the linkage between the two glucose of the dimer.

The high consumption of glucose and other glycolytic intermediates in various cultures, demonstrates the presence of an active glycolytic pathway in cultured insect cells. However, since lactate and pyruvate are unable to support Sf9 cell growth (Reuveny *et al*., 1992) the glucogenesis pathway may not be operating effeciently to facilitate cell growth in the absence of a glycolytic substrate.

Surcrose utilization, upon glucose depletion, has been reported for the insect cell line *Antheraea euclaypti* (Grace, 1966; Clements & Grace, 1967; Stockdale & Gardiner, 1975). Other reports (eg., Ferrance *et al*., 1993, Bedard *et al*., 1993) have noted that level of surcrose does not vary significantly in Sf9 cell cultures. Because of high concentration of sucrose in the media, low rates of sucrose utilization can not be ruled out. Consumption of sucrose by Sf9 cells during the postinfection period has been reported by Wang *et al*. (1993) which is in contrast with the finding of Wong *et al*. (1994).

Finally, it should be noted that different insect cell lines exhibit different pattern of carbohydrate utilization. For example, in sharp contrast to Sf9 cells, the consumption of sucrose by BM5 cells, in a medium containing glucose, maltose, and surcrose is higher than other carbohydrates (Stravroulakis *et al*., 1991a). Moreover, maltose is not substantially consumed by the BM5 cells (Stravroulakis *et al*., 199la). Furthermore, while Sf9 cells do not accumulate significant amounts of lactate (Ferrance *et al*., 1993; Bedard *et al*., 1993; Wang *et al*., 1993), BM5 cultures accumulate lactate to inhibitory levels (Stravroulakis *et al*., 1991 and b).

Amino acid metabolism

Ferrance *et al*. (1993) have reported that the amino acids aspartate, asparagnine, glutamate, glutamine, and serine are utilized at a much higher rate than required for incorporation to cellular proteins and other structures. Arginine, aspargine, aspartate, glutamate, and glutamine can easily be oxidized to CO_2 via malic and the TCA cycle enzymes. One intriguing issue is whether the energy provided by the metabolism of these amino acids is required for biosynthetic functions or their presence at higher levels stimulate their uptake within a regulatory context somewhat different than a global energy optimization.

A useful index of coupling of the energetic and biosynthetic reactions is the ATP yield coefficient $Y_{x/ATP}$ (g. cells generated/mole ATP produced). The total ATP production and hence the value of the $Y_{x/ATP}$, can be obtained by either monitoring the oxygen uptake rate or the information on the metabolic flow of reactions which procedure the reducing power, NADH and FADH, and the substrate level ATP (section 3.3.d). The theoretical maximum value for this coefficient varies slightly with the nature of the medium but it is close to a value of 30 (Stouthamer, 1979; Stouthamer *et al*., 1990). Ferrance *et al*. (1993) found that for insect cells cultivated in media with high (IPL-41) and low (or more balanced) amino acid content (EMICG), the values of $Y_{x/ATP}$ were 5 and 22 (See Table 1), respectively. This finding signifies a substantially higher degree of coupling of energetic and biosynthetic reactions, in the medium with a more balanced amino acid content.

There is a significant literature, primarily in bacterial systems, in which the discrepancy between the ATP production and consumption, termed maintenance requirements, has been attributed to membrane energization, macromolecule turnover, futile cycles, and possibly other unknown essential cellular functions. Our group has recently questioned such a notion and has demonstrated that a better coordination of primary pathways via media design, compatible with metabolic regulations, leads to an almost complete synergy between ATP production and consumption. This assessment is based on attaining, in a metabolically designed media, $Y_{x/ATP}$ values close to the theoretical maximum value of 30 which ignores maintenance functions (Goel *et al*., 1995 a and b). The significantly higher ATP yield coefficient, in insect cells cultured in a medium with a more metabolically balanced amino acid composition suggests that, similar to bacterial systems, a significantly higher synergy between

Table 1. Summary of overall culture data[1] for experiments with IPL-41 and EMICG

Parameter	IPL-41	EMICG
Final cell density (x 10,000 cells/ml)	474	798
Initial cell density (x 10,000 cells/ml)	32	58
Cell mass formed	2728	4568
Initial Amino Acid[2]	12700	5484
Final Amino Acid[2]	9450	2742
Amino Acid used	3250	2742
Percent amino-acid used	26	50
Initial Glucose	2580	4000
Final Glucose	240	1000
Initial Maltose	1000	0
Final Maltose	0	0
Cell Yield[3] (cell carbon/carbon supplied)	0.20	0.54
Cell yield[3] (cell carbon/used)	0.50	0.84
ATP yield coefficient[4] (g cell/mol ATP)	5	22

[1] Initial and final refer to concentration in, μg/ml, at the time of inoculation and at the end of exponential growth, respectively.
[1] Concentration of individual amino acids were also obtained and used in the flow calculations given in Table 2.
[2] Cell yield were calculated based on a carbon content of 0.45 g/cell.
[3] The ATP yield coefficient was obtained as discussed in section 3.3 d.

ATP production and consumption could be attained in insect cell cultures by appropriate media formation which closely reflects the biosynthetic and energetics demands. Moreover, it highlights the potential of $Y_{x/ATP}$ along with carbon yield (carbon in cells/total carbon consumed) as markers for assessing how far the culture performance is from an optimal value by comparing the $Y_{x/ATP}$ and carbon yield to the theoretical maximum values.

Formation of metabolic by-products

Experiments with different concentrations of glucose in the TNM-FH media unveiled that for the range of glucose concentrations examined (4–24 mM), the final cell density does not increase in proportion with the initial glucose concentration (Bedard *et al.*, 1993). However, glucose is fully consumed in all cases and alanine production is high in cultures cultivated with a high initial glucose concentration. Upon the exhaustion of glucose, ammonia begins to accumulate and the alanine concentration drops. The drop in alanine concentration may indicate consumption of alanine at later stages of growth (Bedard *et al.*, 1993). Moreover, while glutamate consumption was higher in cultures with higher initial glucose concentration, glutamine consumption was higher for the cultures with lower initial glucose concentrations. Furthermore, the ammonia production was lowest in cultures with the highest initial glucose concentration.

Due to the complexity of metabolic reaction networks, it is difficult to obtain a coherent picture of these important observations. However, speculation can be made to the possible routing of the key amino acids and glucose. We suspect that a low level of alanine production in culture with the low initial glucose concentration (4 mM) indicates almost full utilization of glucose to biosynthetic precursors derived from glucose through HMP pathway and those obtained from glycolytic intermediates. This hypothesis is also consistent with the highest level of ammonia production and glutamine consumption as a lower glucose flux could lead to a significantly lower incorporation of glucose carbon to TCA cysle intermediates and thus a higher utilization of amino acids. Relatively higher consumption of amino acids, for fulfilling the energetic requirements, will in turn increase ammonia production. Finally, higher glutamate consumption in cultures with higher glucose concentration may reflect the glutamate conversion to alanine via the alanine transaminase catalyzed reaction. Metabolic flow analysis, discussed next, provide a powerful tool for a more rigorous analysis of metabolic consequences of various feeding strategies.

Metabolic flow analysis

Formulation of pathways and the assumptions

The details of the approach used to avaluate the metabolic flows of the key pathways which provide energy and metabolic precursors is given elsewhere (Ferrance *et al.*, 1993). In this manuscript, a summary of the approach is first presented followed by a more critical discussion of the results than is available in the report by Ferrance *et al.* (1993).

Figure 1 depicts reactions/pseudo-reactions comprising the pathways considered in the analysis of the metabolic flows. Several assumptions were made in

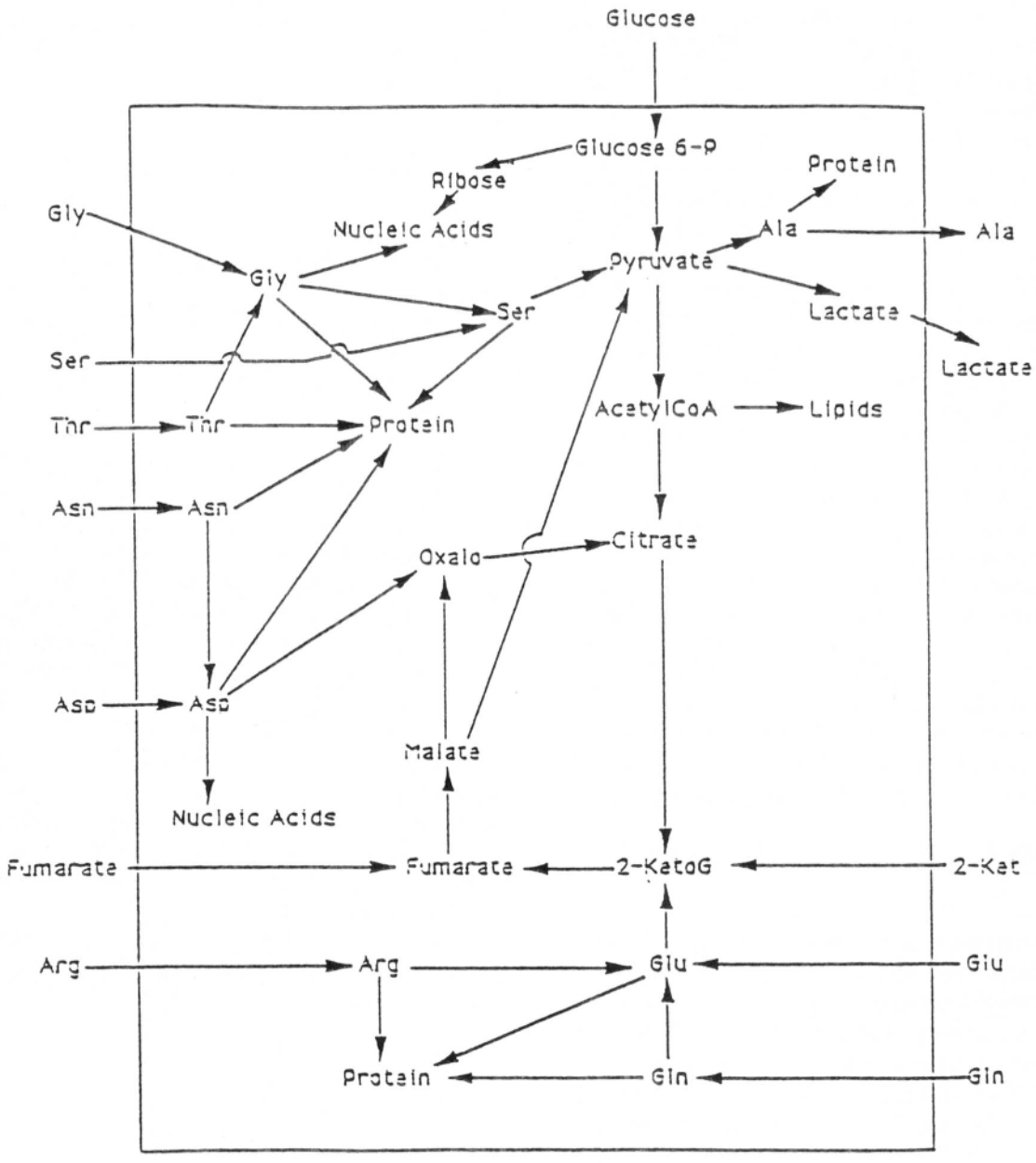

Figure 1. Schematic of the reaction networks used in the analysis of the molar flow of metabolites.

this formulation. First, the HMP pathway is only used for the biosynthetic functions of nucleotides. Secondly, the biosynthetic requirements of amino acids were satisfied by the amino acid transport from the media than their *in vivo* synthesis. Mitsuhashi (1976) studied the amino acids requirement for the PX-58 insect cell line by eliminating one amino acid at a time from the growth medium supplemented with serum having low or negligible amount of free amino acids. The deletion of **α-alanine, β-alanine,** phenylalanine, glutamic acid and glycine one at a time had no adverse effect on cell growth. On the other hand, the deficiency of arginine, asparatic acid, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, proline, and

serine had severe effects on cell growth. For example, the cell proliferation ceased in a flutamine deficient medium. These results support the assumption that the majority of amino acids are not synthesized by insect cells. Moreover, for the amino acids which are not apparently required for growth, the decrease in their culture concentrations during the growth period is in excess of the biosynthetic demands (Ferrance *et al.*, 1993), revealing the preference for uptake over the synthesis.

Thirdly, it was assumed that although, the medium itself contains fatty acids, the lipid requirements are satisfied by *in vivo* synthesis. This assumption was based on reports indicating that *Spodoptera* cells produce, and even excrete fatty acids during growth rather than using the lipids available in the medium (Louloudes *et al.*, 1973) (Some components of the lipid emulsion, however, are needed for cell growth). In the model, the lipid requirements are satisfied by using acetyl CoA as a precursor. The $r_{ACoA-Lip}$ was obtained using the information on the lipid contents of various insect cell lines (Mitsuhashi *et al.*, 1982). Finally, the variation in cell composition was considered to be small. It should be noted that the key fluxes are not sensitive to variations in cell composition. For example 10% variations in the cell's macromolecular composition only marginally affects the evaluated metabolic fluxes of glycolysis and TCA cycle.

Evaluating the metabolic flows

The flows labeled with i's indicate the amount (mmol μl^{-1}) of a component which is taken up by the cells from the medium. For all the amino acids and other components except glutamine, this amount was determined by the change in the medium concentration at the beginning of cultivation and a time at the end of exponential phase of growth. For glutamine, however, the amount uptaken by the cells is obtained by subtracting the amout of glutamine degraded from the value of change in the medium. The flow labeled with p's represent the incorporation of various amino acids to proteins. These values were obtained from the amino acid analysis of cell extract.

In total, there are 45 flow values that must be defined. The values for 27 of them are obtained from experimental measurements and stoichiometry, and the remaining 18 are evaluated from balances conducted around the pools of arginine, glutamine, glutamate, citrate, α-ketoglutarate, asparagine, aspartate, fumarate, malate, oxaloacetate, threonine, glycine, serine, glucose 6-P, ribose, pyruvate, alanine, and acetyl CoA. For example, the balance equations for glucose 6-P and pyruvate are:

$$r_{G6P-Pyr} = 2 \times (r_{G6P} - r_{G6P-NA}), \text{ and}$$

$$r_{Pyr-ACoA} = r_{Ser-Pyr} + r_{G6P-Pyr} + r_{Mal-Pyr} - r_{Pyr-Ala} - r_{Pyr-Lac}.$$

The pseudo-stead-state balances for these components are valid because their intracellular concentrations are insignificant relative to the size of inputs to and outputs from the pools.

Discussion of the molar flow data

A summary of the experimental measurements for cells grown in EMICG and IPL-41 is given in Table 1. The cell yield (g cell carbon/g carbon in carbohydrate and amino acids consumed) was higher for the cells grown in EMICG (0.84) than in IPL-41 (0.50). The amino acids were also used to a larger extent in the EMICG experiments (50%) than the IPL-41 (26%); thus the yield based on total carbon supplied in the medium is significantly higher in the EMICG (0.54) than the OPL-41 (0.20) medium. Table 2 lists the key molar flows for cells grown in both IPL-41 and the EMICG media. The molar flows are presented as the total flow followed by the normalized value, in parenthesis, obtained by dividing the total flow by the cell concentration towards the end of the growth. As the total amount of flucose consumed is similar for the EMICG and IPL-41 geown cells (see the flow of Gluc-G6P), the total flows may also be thought of as normalized per glucose used.

Note that the flow for aspartate to oxaloacetate for the cells grown in EMICG is negative. This negative flow implies a lower uptake of aspartate than needed for the biosynthesis of amino acids and nucleotides. Since it is highly likely that yeastolate contains some nucleotide derivatives and bases this flow may become a small positive value if the possible contribution of yeastolate to the precursor pools of nucleotide pathways was available and accounted for in the model. Of course the negative flow for aspartate to oxaloacetate can not be observed in the IPL-42 medium in which most of the amino acids are far in excess of biosynthetic requirements. The negative flow for alanine, fumarate, and α-ketoglutarate signifies their secretion to the culture. Overall, the flows for the cells grown in EMICG indicates that the efficient incorporation of carbon to biomass in this medium results from a tight coupling

Table 2. Flows in millimolar and (mmole/g-cell) for reactions in Figure 1

Flow	IPL-41	EMICG
i Arg	2.08(0.71)	3.33(0.73)
i Gln	1.21(0.41)	2.68(0.59)
i GlU	7.21(2.46)	4.63(1.02)
i Asn	4.72(1.61)	1.78(0.39)
i Asp	4.39(1.50)	2.19(0.48)
i Thr	1.22(0.42)	0.99(0.22)
i Gly	0.87(0.30)	2.84(0.62)
i Fum	–0.71(0.24)	0(0)
i KG	–0.14(–0.047)	0(0)
i Ser	2.18(0.75)	3.42(0.75)
i Ala	–7.76(–2.65)	–4.56(–1.00)
p Arg	0.51(0.17)	0.77(0.17)
p Gln	0.54(0.18)	0.82(0.18)
p Glu	0.53(0.18)	0.82(0.18)
p Asn	0.50(0.17)	0.77(0.17)
p Asp	0.50(0.17)	0.77(0.17)
p Thr	0.45(0.15)	0.68(0.15)
p Gly	0.81(0.27)	1.22(0.27)
p Ser	0.49(0.17)	0.77(0.17)
p Ala	0.88(0.30)	1.36(0.30)
Asp-NA	1.70(0.58)	2.51(0.58)
Gly-NA	0.85(0.29)	1.32(0.29)
G6P-Rib	1.70(0.58)	2.63(0.58)
Rib-NA	1.70(0.58)	2.63(0.58)
ACoA-Lip	10.98(3.72)	16.89(3.72)
Pyr-Lac	0(0)	0(0)
Gln-Glu	0.67(0.23)	1.82(0.40)
Glu-KG	8.92(3.05)	8.14(1.79)
Arg-Glu	1.57(0.54)	2.56(0.56)
Asn-Asp	4.22(1.44)	0.97(0.21)
Asp-Oxalo	6.41(2.19)	–0.48(–0.11)
Mal-Pyr	14.48(4.95)	7.66(1.68)
Thr-Gly	0.77(0.26)	0.26(0.06)
Gly-Ser	–0.02(–0.01)	0.37(0.08)
Ser-Pyr	1.67(0.57)	2.99(0.66)
Gluc-G6P	18.84(6.44)	16.61(3.64)
G6P-Pyr	34.28(11.72)	27.54(6.04)
Pyr-Ala	8.64(2.95)	5.99(1.31)
Pyr-AACoA	41.79(14.28)	32.21(7.06)
ACoA-Cit	30.81(10.53)	13.80(3.03)
Oxalo-Cit	30.81(10.53)	13.80(3.03)
Cit-KG	30.81(10.53)	13.80(3.03)
KG-Fum	39.59(13.53)	21.94(4.81)
Fum-Mal	38.88(13.29)	21.94(4.81)
Mal-Oxalo	24.40(8.34)	14.28(3.13)

The numbers enclosed in parentheses are normalized flow (mmole/ g-cell).

of the biosynthetic and energetic pathways as assessed by lower values of alanine production (See the normalized values in the parentheses in Table 2), glucose and amino acid uptakes, and the higher values of the cell and ATP yield coefficients.

The molar flow data may also be used to answer the following questions.

Is there a fully operational TCA Cycle? The $r_{ACoA-Cit}$ (Table 2) for the cells grown in IPL-41 (a medium with high amino acid content) and EMICG (a medium with lower amino acid content) is 10.5 and 3.03, respectively. The molar flows were obtained making the assumption that fatty acid requirements are satisfied entirely from acetyl CoA. If the fatty acid requirements are partially satisfied from the uptake of medium components, then the $r_{ACoA-Cit}$ will be larger the value given in the Table 2. The high value of $r_{ACoA-Cit}$ for cells grown in IPL-41 and the lower value of the sum of $r_{Mal-Pyr} + r_{ser-Pyr}$ than the $_{ACoA-Cit}$ indicate that the Sf9 cells possess a fully operational TCA cycle and glucose carbon enters the TCA cycle.

How to reduce the overproduction of alanine? The combination of lower specific glucose and amino acid uptakes to EMICG as compared to IPL-41 media (see the number in parenthesis in Table 2) leads to a significantly lower specific alanine secretion for the cells grown in EMICG. The formation of large amounts of ammonia, produced as part of the amino acid catabolism, point to the production of alanine as a possible sink for ammonia. That is the presence of high levels of amino acids in the media such as IPL-41 could lead to higher than "needed" uptake rates which in turn results in excessive alanine formation. Thus, formulating a balanced medium with an amino acid composition closely reflecting the energetic and biosynthetic demands may lead to a drastic decrease in alanine production. Such formulations will be greatly aided by a better understanding of the interrelations between glucose and amino acid catabolism.

Oxygen consumption and carbon dioxide production. Several reports have indicated that cultures in which dissolved oxygen concentrations were maintained at is relatively high levels attained significantly higher culture productivity (greater than 5% of saturation). A nice review of the effect of dissolved oxygen and oxygen uptake rate on culture productivity is available in a report by Taticek *et al.* (1995). A better understanding of the nature of shear sensitivity of insect cells and

design of reactor configurations that allows for a higher oxygen transport rate would lead to more productive insect cell cultures. The issue of oxygen requirements, however, has not been viewed from a physiological stand pont. An improved knowledge of the physiological and nutritional requirement will facilitate the design of productive culture media or feeding strategies for reducing the oxygen requirements. The oxygen uptake can be regulated by controlling the production of the reducing power NADH. The tight coupling of energetic and biosynthetic reactions can significantly reduce the NADH generation. An instructive example entailing a comparison of the NADH generation in a medium with a low aino acid content (EMICG) and a medium with a high amino acid content (IPL-41 is duscussed below.

The relation between oxygen consumption and the NADH production is $Q_{02} = 1/2\ r_{NADH}$. Based on the formulation shown in Figure 1, NADH production is r_{NADH} (mmole/g-cell) = $r_{G6P-Pyr} + r_{Pyr-ACoA} + r_{Cit-KG} + 2r_{KG-Fume} + r_{Mal-Oxalo} - (18 - r_{Mal-Pyr} - 2rr_{G6P-Rib})$. It is assumed that FAD is equivalent to NADH with respect to ATP generation. The first five terms reflect the NADH production and the last term accounts for the NADH conversion to NADPH by the action of transhydrogenases. The NADPH g^{-1} cell required for biosynthetic reactions was assumed to be 18 mmole/g-cells which is the value reported for bacterial systems. The $r_{Mal-Pyr}$ and $r_{G6P-Rib}$ are accounting for the NADPH synthesized in those reactions. Thus, $(18 - r_{Mal-Pyr} - 2r_{G6P-Rib})$ represents the net requirement of NADPH provided for by the action of transhydrogenases. It should be noted that we have shown that if the GMP pathway, instead of the transhydrogenases reactions as used for production of NADPH, r_{NADH} will not be significantly different.

Using the above equation, the oxygen consumption for the EMICG medium and the IPL-41 medium are 13 and 60 mmol g^{-1} cell respectively. Thus, a drastic reduction in oxygen requirements (on per gram cell basis) isd attained in a medium with a composition more closely matched to the biosynthetic and energetic requirement.

There is very little information on the effect of dissolved CO_2; we have just begun to examine how the dissolved CO_2 affects the culture productivity. The amount of evolved CO_2 can be calculated in insect cell by measuring the flux of the reactions producing CO_2. Ferrance *et al.* (1993) calculated the evolved CO_2 by evaluating the flux of reactions coupled to CO_2 formation ($r_{Pyr-ACoA} + r_{Cit-KG} + r_{KG-Fum} + r_{Mal-Pyr} + r_{G6P-Rib}$) and found it to be very close to the measured value.

Evaluation of the ATP yield coefficient. The flows given in Table 2 can also be used to calculate the ATP yield coefficient ($Y_{x/ATP}$, g cell mol^{-1} ATP). The ATP production r_{ATP} (mmol g^{-1} cell = 3 $[(r_{G6P-Pyr} + r_{Pyr-ACoA} + r_{Cit-KG} + 2r_{KG-Fum} + r_{Mal-Oxalo}) - (18 - r_{Mal-pvr} - 2r_{G6P-Rib})] + 2r_{G6P-Pyr}) - r_{Gluc-G6P} - r_{G6P-Rib})$. The terms in bracket represent the NADH production (coupled to ATP formation) as discussed in ther previous section. The value of 3 is the P/O ration (the number of ADPs phosporylated per electron pair transferred to oxygen). The last three terms represent ATP produced/consumed via substrate level phosphorylation. Using the above equation, the value of $Y_{x/ATP}$ can be obtained as: $Y_{x/ATP} = 1000/r_{ATp}$. The values of $Y_{x/ATP}$ for cells grown in TPL-41 and EMICG medium were given in Table 1, as 5 and 22, respectively.

Post infection metabolism

Several groups have examined the consumption of glucose, glutamine and other amino acids, alanine production, and oxygen uptake during the post-infection period (Weiss *et al.*, 1990; Kamen *et al.*, 1991; Wang *et al.*, 1993; Henseler & Agathos, 1994; Wong *et al.*, 1994). Despite multitudes of factors affecting the post-infection metabolism such as variations in medium composition, the time of infection, multiplicity of infection, etc., some common features may have emerged from the recent metabolic studies. First, as noted, maintaining a relatively high dissolved oxygen concentration leads to a higher recombinant protein productivity (Scott *et al.*, 1992; Wang *et al.*, 1993). Secondly, increased culture productivity is attained by replacement or dilution of spent medium, at the time of infection, with fresh medium (Caron *et al.*, 1990; Lazarte *et al.*, 1992; Lindsay & Betenbaugh, 1992; Wang *et al.*, 1993). Thitdly, supplementing the culture with glucose and glutamine at the time of infection may somewhat enhance the concentration of the recombinant protein (Wang *et al.*, 1993). Finally, supplementation of yeastolate along with glucose and glutamine coupled with media replacement substantially elevates the rate of protein production (Reuveny *et al.*, 1993). A recent report has revealed that Sf9 cells infected at a high cell number density of $7x10^6$ ml^{-1} attains high recombinant productivity when supplemented, without media change or a significant dilution at the time

of infection, with yeastolate and a mixture of all other amino acids (Bedard *et al*., 1994). This finding may suggest that low productivity for Sf9 cultures infected at the late exponential phase (e.g. Caron *et al*., 1990; Lazarte *et al*., 1992; Reuveny *et al*., 1993) reflects a nutrient limitation rather than the build-up of toxic etabolic by-products. This report also support the observation that cell-cell contact inhibition at higher cell densities appears to be insignificant for Sf9 and Sf21 cells (Wickham *et al*.., 1992).

The effect of yeastolate on protein productivity may reflect the supply of a limited nutrient by the mixture. Since addition of vitamins and lipid components does not produce productivity enhancement achieved by yeastolate (Reuveny *et al*., 1993), we suspect that some other essential compound(s), perhaps a component of the nucleotide pathways is supplied by yeastolate. Nucleotides serve as building blocks for the synthesis of nucleic acids, structural moiety of coenzymes, metabolic regulators and signal molecules, ans are critical elements of energy metabolism. Because of the significant role of nucleotides in cellular regulation and DNA replication, efforts aimed at a better understanding of nucleotide metabolism of insect cells will be highly desirable.

Future Research

Media without yestolate

Despite limited knowledge on insect cell metabolism during pre- and post infection, significant progress has been made. The original media for cultivating insect cells contained serum or hemolymph. The presence of serum makes purification of the target protein more difficult and leads to variation in product yields due to the variation in the composition from lot to lot. It also significantly complicates detailed metabolic studies. Work on replacing serum with other components has yielded serum-free media. The serum-free media contain sugars, salts, amino acids, organic acids, vitamins, trace elements, and ultrafiltered yeastolate. Although it is feasible to gain some metabolic insights using insect cells cultivated in yeastolate containing media, its substitution with chemically defined components will vastly enhance the range and the scope of such studies. The chemically defined medium will also ensure reproducibility of optimized process conditions.

Wilkie *et al*. (1908) have claimed a chemically defined medium (CDM) lacking yeastolate. We prepared this medium from the individual components. Several attempts to subculture the cells in CDM proved unsuccessful. It was found that al though the cells will grow in CDM for the first subculture, they do not survive past one subculturing in shake flasks. Successful subculturing of the cells became possible only when the CDM was supplemented with yeastolate. However, recently we have made progress in substituting yeastolate with chemically defined components. These findings will be communicated at a later time.

Performance of metabolite routing studies

We have shown that significant insight may be gained by the stoichiometry-based analysis of the primary metabolic pathways. More metabolic flow studies under different nutritional conditions will expand the current metabolic knowledge of insect cells. However, for elucidating regulatory and flux routing details, these studies must ultimately be coupled to measurements on concentrations of intracellular meabolites and enzymes, and tracer compound studies. Such experiments, currently underway in our laboratory would supplant the fluxes identified from using overall balances and observables by filling in gaps and eliminating uncertainties regarding the glucose-6-phosphate, pyruvate, and malate branch points. The metabolic routing studies with radiolabelled substrates is also critical for an improved understanding of post-infection metabolism.

References

Bedard C, Tom R & Kamen A (1993) Growth, nutrient consumption and end-product accumulation in Sf9 and BTI-EAA insect cell cultures: Insights into growth limitation and metaboolism. Biotechnology Progress 9: 615–624.

Bedard C, Kamen A, Tom R & Massie B (1994) Maximization of recombinant protein yield in the insect cell/baculovirus system by on-time addition of nutrients to high-density batch cultures. Cytotechnology 15: 129–138.

Caron AW, Archambaust J & Massie B (1990) High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. Biotechnology and Bioengineering 36: 1133–1140.

Cho T, Shuler ML & Granados RR (1989) Current developments in new media and cell culture system for the large-scale production of insect cells. In: Advances in Cell Culture. Vol. 7 (pp. 261–277) Academic Press, New York.

Clements AN & Grace TDC (1967) The utilization of sugars by insect cell in culture. Journal of Insect Physiology 13: 1327–1332.

Fraser MJ (1989) Expression of eucaryotic genes in insect cell culture. In vitro cellular and developmental biology 25 3: 225–235.

Ferrance JP, Goel A & Ataai MM (1993) Ultilization of glucose and amino acids in insect cell cultures: Quantifying the metabolic flows within the primary pathways and medium development. Biotechnology and Bioengineering 542: 697–707.

Goel A, Domach MM & Ataai MM (1995a) A Novel Dual feeding Strategy for Suppression of Acid formation in Bacteria: Lowering of the Glycolysis Flux. Biotechnology Progress in press.

Goel A, Domach MM, Hanley W & Ataai MM (1995b) Coordination of Glycolysis and TCA Cycle Reaction Networks: Citrate-Glucose Co-metabolism Eliminate Acids and reveals Potential Metabolic Engineering Strategies. Ann. NY. Acad. Sci., in press.

Grace TDC & Brzostowski HW (1966) Analysis of the amino acids and sugars in an insect culture medium during cell growth. Journal of Insect Physiology 12: 625–633.

Hensler WT & Agathos SN (1994) Evaluation of monitoring approaches and effects of culture conditions on recombinant protein production in baculovirus-infected cells. Cytotechnology 15: 177–186.

Hink WF, Thomsen DR, Davidson DJ & Castellino FJ (1991) Expression of three recombinant proteins using baculovirus vectors in 23 insect cell lines. Biotechnology Progress 7: 9–14.

Kamen AA, Tom RL, Caron AW, Massie B & Archambault J (1991) Culture of insect cells in a helical ribbon impeller bioreactor. Biotechnology and Bioengineering 38: 619–628.

Lazarte JE, Tosi PF & Nicolau C (1992) Optimization of the production of full-length rCD4 in baculovirus-infected Sf9 cells. Biotechnology and Bioengineering 40: 214–217.

Licari P & Bailey JE (1992) Factors influencing recombinant protein yields in an insect cell-baculovirus system: multiplicity of infection and intracellular protein degradation. Biotechnology and Bioengineering 39: 614–618.

Lindsay DA & Betenbaugh MG (1992) Quantification of cell culture factors affecting recombinant protein yields in baculovirus-infected cells. Biotechnology and Bioengineering 39: 614–618.

Louloudes SJ, Vaughn JL & Dougherty KA (1973) Fatty acid profiles of cells from cell line IRPL-21 (*Prodoptera frugiperda*) and of the tissue culture medium after repeated use. In Vitro 8: 473–479.

Lynn DE & Hink WF (1980) Comparison of Nuclear Polyhedrosis Virus replication in five lepidopteran cell lines. Journal of Invertebrate Pathology 35: 234–240.

Mathews CK & Holde VEK (1990) Biochemistry. The Benjamin/Cummings Publishings, California.

McIntosh AH & Grasela JJ (1994) Expression of b-galactosidase and luciferase in insect cell lines infected with a recombinant AcMNPV. In Vitro Cell Development Biology 30A: 275–278.

Mitsuhashi J (1976) Insect cell line: amino acid utilization and requirements. In: Kurstak E & Maramorosch K (eds) Invertebrate Tissue Culture. (pp. 257–262) Academic Press, London.

Mitsuhashi J, Makasone S & Hori Y (1985) Total Fatty acids of some insect cell lines. App. Ent. Zool. 20: 8–12.

Murhammer DW (1991) The use of insect cell cultures for recombinant protein sysnthesis: Engineering aspects. Applied Biochemistry and Biotechnology 31: 283–310.

O'Reilly DR, Miller LK & Luckow VA (1992) Baculovirus Expression Vectors: a laboratory manual. W. H. Freeman and Company, New York.

Reuveny S, Kemp CW, Eppstein I & Shiloach J (1992) Crbohydrate metabolism in insect cell cultures during cell growth and recombinant protein production. In: Pederson H, Mytharasan R & Dibasic D (eds) Biochemical Engineering. Vol. 1. VII (pp. 230–237) New York Academy of Sciences.

Reuveny S, Kim YJ, Kemp CW & Shiloach J (1993) Production of recombinant proteins in high-density insect cell cultures. Biotechnology and Bioengineering 42: 235–239.

Scott TRI, Blanchard JH & Ferguson CHR (1992) Effects of oxygen on recombinant protein production by suspension cultures of Spodoptera frugiperda (Sf9) insect cells. Enzyme Microbiology Technology 14: 798–804.

Stravroulakis DA, Kalogerakis N & Behie LA (199la) Kinetic data for the BM-5 insect cell line in repeated-batch suspension culture. Biotechnology and Bioengineering 38: 116–126.

Stavroulakis DA, Kalogerakis N & Behie LA (1991b) Growth characteristics of a *Bombyx mori* Insect cell line in stationary and suspension culture. The Canadian Journal of Chemical Engineering 69: 457–464.

Stockdale H & Gardiner GR (1976) Ulilization of some sugars by a line of *Trichoplusia NI* cells. In: Kurstak E & Maramorosch K (eds) Invertebrate Tissue Culture, (pp. 267–274) Academic Press, London.

Stouthamer AH (1979) The Search for Correlation Between Theoretical and Experimental Yields. In: Ouayle JR (ed.) Microbial Biochemistry. University Park Press, baltimore.

Stouthamer AH, Bulthuis BA & Verseveld HWV (1990) Energetics of Growth at Low Growth Rates and its relevance for the Maintenance Concept. In: Poole RK *et al*. (eds) Microbial Growth and Dynamics. Oxford University Press.

Taticek RA, Hammer DA & Shulker MA (1995) Overview of issues in bioreactor design and scale-up. In: Shuler ML *et al*., (eds) Baculovirus expression systems and biopesticides. (pp. 131–174) Wiley-Liss Inc., New York.

Wang MY, Vakharia V & Bentley, WE (1993) Expression of epoxide hydrolase in insect cells: A focus on the infected cell. Biotechnology and Bioengineering 42: 240–246.

Wang MY, Kwong S & Bentley WE (1993) Effects of oxygen/glucose/glutamine feeding on insect cell baculovirus protein expression: A study on the Expoxide Hydrolase Production. Biotechnology Progress 9: 355–361.

Weiss SA, Gorfien A, Fike R, DiSorbo D & Jayme D (1990) Large scale production of proteins using serum-free insect cell culture. In: Ninth Australian Biotechnology Conference proceedings, Biotechnology: The science and business. (pp. 230–231) Gold Coast Australia.

Wickham TJ, Davis T, Granados RR, Shuler ML & Wood HA (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnology Progress 8: 391–396.

Wickham TJ,. Davis T, Granados RR, Hammer DA, Shuler ML & Wood HA (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnology Letters 13: 483–188.

Wilkie GEI, Stockdale H & Prit S V (1980) Chemically defined media for production of insect cells and viruses *in vitro.* develop. Biol. Standard. 46: 29–37.

Wong TKK, Nielsen LK, Greenfield PF & Reid S (1994) Relationship between oxygen uptake rate and time of infection of Sf9 insect cells infected with a recombinant baculovirus. Cytotechnology 15: 157–167.

Wood AH (1995) Development and testing of genetically improved baculovirus insecticides. In: Shuler ML *et al*., (eds) Baculovirus expression system and biopesticides. (pp. 91–102) Wiley-Liss, New York.

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Insect cell cultivation: growth and kinetics

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Introduction

The baculovirus-insect cell expression system has emerged as a fast and powerful tool for the production of numerous heterologous proteins which are a prerequisite e.g. for initiating random screening programs aimed at identifying low-molecular-mass non-proteinaceous drug substances, for performing X-ray crystallographic structure/function studies and rational drug design, and also for establishing "proof of principle" in animal studies of human diseases. If any of the above mentioned applications requires sufficiently large amounts of intact biologically active protein it will become necessary to carry out process optimization studies.

This review examines the progress made towards the cultivation of insect cells in controlled bioreactors with particular reference to the growth kinetics and protein expression under different physicochemical conditions and the published data on nutrient and by-product metabolic quotients during growth and infection. The focus is on the recent literature published until the end of 1994.

Suspension versus immobilized cultures

A limited number of publications over the past years dealt with the growth of insect cells in immobilized culture systems (Agathos *et al.*, 1990; Lazar *et al.*, 1987; Archambault *et al.*, 1994; Wickham & Nemerow, 1993; Kompier *et al.*, 1991; King *et al.*, 1989; Chung *et al.*, 1993). As the initial difficulties of cultivating insect cells in suspension using stirred tank or airlift bioreactors with submerged aeration or microsparging have been overcome (see below) and most insect cell lines can be adapted to grow in suspension, further research into such immobilized culture systems would only seem appropriate if strictly adherent cell lines showed greatly enhanced potential with respect to product yields and quality. Above all, the recovery of intracellular protein products may be difficult to achieve at large scale. In the following only suspension cultures will be considered.

Growth rates and maximal cell concentrations for suspension cells

Effect of different media and media supplements including serum

The kinetics of insect cell growth have by now been evaluated in quite an extensive number of investigations in controlled bioreactors. An overview of these is presented in Table 1. Most studies make use of insect cell lines derived from Spodoptera frugiperda, i.e., Sf9 or Sf21, and results are thus well comparable. Cell growth has been examined in airlift and stirred tank bioreactors with working volumes up to 150 l whilst maintaining oxygenation by bubble-free aeration, orifice sparging and microsparging or a combination of methods. Similar growth characteristics have been reported for cells cultivated in serum containing and serum-free media if shear protective agents like Pluronic F–68 are included in media formula-

tions. Optimal Pluronic F–68 concentration have been found to range from 0.1 to 0.3% (w/v) (Murhammer & Goochee, 1988; Zhang *et al*., 1994; Caron *et al*., 1990). The incorporation of undefined hydrolyzates like yeastolate, lactalbumin hydrolyzate or Primatone RL in the culture media has a marked influence on maximum cell densities. Limitations in any of the commonly quantitated medium components like carbohydrates, amino acids or lipids (Schmid, unpublished results) can be averted by increasing the initial concentrations or nutrient feeding over the course of the fermentation. Reported specific growth rates (μ) for Sf9 and Sf21 cells are in the range of 0.67–0.83 day^{-1}, which corresponds to average population doubling times of 20 to 25 hr. Maximum cell densities approach $10 \times 10E6$ viable cells ml^{-1} without additional feeding of nutrients (Schlaeger *et al*., 1993). With feeding strategies (Nguyen *et al*., 1993; Schlaeger *et al*., 1992) or cell retention and medium perfusion (Caron *et al*., 1994; Deutschmann & Jäger, 1994) biomass yields of up to $50 \times 10E6$ cells ml^{-1} have been obtained.

In most cases insect cell cultures are, however, infected with recombinant baculovirus preparations during the early part of the exponential growth phase and at viable cell concentrations well below the maximum cell densities that are indicated above. The challenge still remains to maintain a high level of protein expression (i.e., identical specific productivity), when infecting cultures at high cell density, and thus to increase the space-time-yield without complete medium exchanges before infection or continuous medium perfusion during infection as both of these approaches are difficult to perform in large scale operations.

Effect of hydrodynamic environment

Even in the recent literature there have been reported conflicting results with regard to the detrimental effects of sparging and microsparging on insect cell growth and culture viability. For example, Jain *et al.* (1991b) and Caron *et al.* (1990) reported identical growth characteristics for Sf9 cells using media supplemented with Pluronic F–68 as shear protectant regardless of whether surface aeration, orifice sparging, microsparging or bubble-free silicon tube gassing were employed for oxygenation purposes. On the other hand, Blanchard & Ferguson (1992) observed a negative effect of air sparging (compared to silicon tube gassing) on the viability of uninfected Sf9 cells using SF900 serum-free medium with 0.1% (w/v) Pluronic F–68. The extend of damage caused by submersed aeration or microsparging (if any) depends, among other things, on the hydrodynamics of the individual bioreactor, the (micro)sparger type and pore size, the gas flow-rate, and the type and concentration of added shear protectants. Many groups therefore now routinely use either stirred tank or airlift bioreactors at the pilot scale for insect cell cultivations. Guillaume *et al.* (1992) at Rhone-Poulenc Rorer found maximum cell densities and specific growth rates of Sf21 cells as well as the time-course of infection with 2 recombinant baculovirus constructs comparable for 2, 10 and 1501 stirred tank reactors when using pure oxygen sparging for DO control.

Recently, researchers at Merck (Junker *et al*., 1994) reported on the use of a modified 75 1 microbial fermenter for insect cell cultivations. At Hoffmann-La Roche we have used 25 and 75 1 airlift as well as 1501 stirred tank reactors for the production of a variety of recombinant proteins from insect cells over the past years (Schlaeger *et al*., 1992; Schlaeger *et al*., 1995; Schmid *et al*., 1994). Airlifts are operated at 0.03–0.07 VVM with additional microsparging at high cell densities. The 150 1 vessels were conventional microbial bioreactors (Chemap) equipped with either a sail-type Teflon impeller or Rushton turbines. Oxygenation and pH control were achieved by orifice sparging of an air/oxygen/nitrogen/carbon dioxide mixture via a gas blending unit fitted with mass-flow controllers. Sf9 growth and expression of recombinant IFNγ receptors was identical to results obtained in airlift reactors (Schmid *et al*., 1994 and unpublished results).

The effects of hydrodynamic forces on insect cells in suspension leading to increased cell damage or death are discussed in detail by Chalmers (see pp. 163–171, this volume).

Effect of dissolved oxygen concentration on growth characteristics

Despite a number of publications that report on the cultivation of insect cells under controlled dissolved oxygen (DO) conditions (e.g., Weiss *et al*., 1989; King *et al.* 1992; Maiorella *et al*., 1988; Caron *et al*., 1990; Wong *et al*., 1994; Kamen *et al*., 1991; Scott *et al*., 1992; Blanchard & Ferguson, 1992; Reuveny *et al*., 1992 and 1993; Lazarte *et al*., 1992; Nguyen *et al*., 1993; Guillaume *et al*., 1992) the effects of different DO concentrations on cell growth rates and maximum viable cell concentrations have only been evaluated in a limited number of bioreactor studies (Table 2).

Table 1. Growth characteristics of various insect cell lines in controlled bioreactors

Type of bioreactor	Working volume (l)	Cell line	Culture medium	Agitation/ Aeration	Physicochemical conditions	Specific growth rate μ (day^{-1})	Maximum cell counts (cells ml^{-1})	Reference
Airlift	21	S. frugiperda (Sf9)	IPL–41 with 10% FBS or serum-free	0.02 VVM	DO 20%	0.67–0.83	5.0 × 10E6	Maiorella *et al.*, 1988
STR, cell retention	1.4, 2, 6	S. frugiperda (Sf21)	IPL–41 with 5% FBS	rpm 40, bubble-free aeration	DO 40–100%	0.39–0.60	1.7–6.6 × 10E6 55 × 10E6 with perfusion	Deutschmann & Jäger, 1994
STR	4, 11	S. frugiperda (Sf9)	TNM-FH with 10% FBS IPL–41 serum-free	60–100 rpm, surface aeration	DO 30–40% no pH control	0.69–0.83 0.69–0.76	5 × 10E6 5.5 × 10E6	Caron *et al.*, 1990; Kamen *et al.*, 1991
STR, cell retention	4	S. frugiperda (Sf9)	TNM-FH with 10% FBS	rpm 70–80, microsparging	DO 30% no pH control	0.74 (batch), 0.36 (perfusion)	12–15 × 10E6 with perfusion	Caron *et al.*, 1994
STR	1.5	S. frugiperda (Sf9)	TC100 5% FBS IPL–41 5% FBS IPL–41 serum-free	rpm 50, bubble-free aeration	DO 40% pH 6.2	–	2.3 × 10E6 5.5 × 10E6 5.6 × 10E6	Fertig *et al.*, 1993
STR	2, 10, 150	S. frugiperda (Sf21)	EX-CELL 400 SFM	rpm 45, microsparging	DO 40% pH 6.2	0.65–0.79	5 × 10E6	Guillaume *et al.*, 1992
STR	2, 12	S. frugiperda (Sf9)	EX-CELL 400 SFM IPL–41 serum-free	rpm 200, 0.01 VVM, orifice sparging	DO 30% pH 6.3	0.83	3 × 10E6 12 × 10E6 with feeding	Nguyen *et al.*, 1993
Airlift	14	S. frugiperda (Sf9, Sf21)	SF–900 SFM	0.025–0.06 VVM	DO 10–20% no pH control	0.69	8 × 10E6	Wu *et al.*, 1992; King *et al.*, 1992
STR	1.5	S. frugiperda (Sf9)	IPL–41 with 5% FBS SF900II SFM	rpm 120, sparging	DO 30% pH 6.2	0.48 0.67	2–5 × 10E6 5–6 × 10E6	Power *et al.*, 1992; Power *et al.*, 1994
Airlift	10	S. frugiperda (Sf9, Sf21)	EX-CELL 400 SFM	0.02–0.04 VVM	DO 10–20% pH 6.2	0.83	2.9 × 10E6	Weiss *et al.*, 1989
Airlift	23	T. ni (BTI-Tn–5B1–4)	SF–1 with 1% FBS or serum-free	0.05 VVM and microsparging	DO 30% pH 6.1–6.3	0.82	4.5–6 × 10E6 8 × 10E6 with feeding	Schlaeger *et al.*, 1995
Airlift STR	23, 60 120	S. frugiperda (Sf9, Sf21)	IP301 or SF–1 with 1% FBS or serum-free	0.05 VVM, microsparging rpm 25–40, 0.02 VVM, ring sparger	DO 30–60% pH 6.1–6.3 DO 30% no pH control	0.67–0.76	9–13 × 10E6 depending on feeding strategy	Schlaeger *et al.*, 1993; Schlaeger & Schmid, unpublished results
STR Airlift	1.5 0.6	S. frugiperda (Sf9)	TNM-FH with 5% FBS	rpm 200, sparging 0.005 VVM	DO 50% no pH control	0.69–0.88 0.78–0.89	2–2.5 × 10E6	Murhammer & Goochee, 1988
STR	5	S. frugiperda (Sf9)	IPL–41 with 10% FBS ICSF-WB SFM	rpm 80, orifice sparging	DO 65% pH 6.2	0.67–0.88 0.69–0.88	4–5 × 10E6 4–5 × 10E6	Reuveny *et al.*, 1992
STR	8, 18	S. frugiperda (Sf9)	IPL–41 with 2% FBS	bubble-free aeration or microsparging	DO 10–110%	0.48–0.65	4–5 × 10E6	Jain *et al.*, 1991a, b
STR	1.5	B. mori (Bm5)	IPL–41 with 10% FBS	rpm 60–85, sparging	DO 10–60% pH 6.3	0.46	3.6–4.8 × 10E6	Zhang *et al.*, 1994; Zhang *et al.*, 1993
STR	100	S. frugiperda (Sf9)	IPL–41 with 10% FBS	rpm 35, microsparging	DO 30% no pH control	0.69–0.83	1.5 × 10E6	Barkhem *et al.*, 1992
STR	1	S. frugiperda (Sf9)	SF900II SFM	rpm 140, surface aeration	DO 50% no pH control	0.74	7 × 10E6	Wong *et al.*, 1994

VVM = volumes of gas per culture volume and minute;
STR = stirred tank reactor;
SFM = serum-free medium.

Table 2. Bioreactor studies examining the effect of different dissolved oxygen concentrations on the growth kinetics of insect cells

Cell line	DO setpoint (% air saturation)[a]	Type and size of bioreactor	Reference
Sf21	40, 70, 100	1.2 l stirred tank, bubble-free aeration	Deutschmann & Jäger, 1994
TN–368	15, 50, 100, >100	2–3 l stirred tank, sparging	Hink & Strauss, 1980; Hink, 1982
Sf9	10, 65, 110	18 l stirred tank, microsparging	Jain *et al.*, 1991a and 1991b
Sf9	20, 40, 60, 80	1.5 l stirred tank, bubble-free aeration	Klöppinger *et al.*, 1990
Sf9	30–60	23 and 60 l airlift, 120 l stirred tank, orifice sparging	Schlaeger & Schmid, unpublished results
Sf9	5, 10, 50, 100	0.25 l stirred tank, surface aeration	Hensler & Agathos, 1994
Bm5	10, 20, 30, 40, 60	1.5 l stirred tank, CelliGen	Zhang *et al.*, 1994

[a] Optimal values that gave highest specific growth rates and cell densities are underlined.

In early studies Hink & Strauss (1980) and Hink (1982) examined the growth characteristics of the Trichoplusia ni TN–368 cell line in sparged stirred tank reactors with working volumes of 2 to 3 1. The specific cell growth rates were found to be similar at all DO levels (maximum growth rate 1.19 day^{-1}). However, cells cultivated at 15% DO were vacuolated at 120 hr, this being followed by a rapid decrease in cell numbers. Cells maintained at DO > 100% exhibited lower maximum cell densities. Klöppinger *et al.* (1990) investigated the effects of different DO setpoints (20, 40, 60 and 80%) on Sf9 cell growth in batch cultures using a 1.5 1 stirred tank bioreactor with bubble-free aeration. Maximum specific growth rates ($\mu = 0.76\ day^{-1}$) were measured for DO levels of 20 and 40%. At 60 and 80% DO these growth rates were only reduced by ~10%. Using TC100 medium supplemented with 10% FBS a maximum viable cell concentration of 2.3 × 10E6 cells ml^{-1} was observed at 40% DO in this series of experiments. Similar experiments were also performed by Jain *et al.* (1991a, b) in an 18 1 stirred tank reactor equipped with 2 μm pore size microspargers. The levels of DO in the culture medium had a significant effect on the growth rate of cells. At 10 and 110% DO the specific growth rates were ca. 25% lower than at 65% DO ($\mu = 0.65\ day^{-1}$). The authors speculated that at low DO cells probably were oxygen-starved and at high DO were experiencing oxygen toxicity effects. Under all three conditions no differences were found for cell viabilities (ca. 98%). This seems to imply that the reduced growth rates of cells are not the result of increased cell death but a direct consequence of the DO concentration in the culture medium. At Hoffmann-La Roche we have consistently observed maximum specific growth rates of $0.73 \pm 0.03\ day^{-1}$ for Sf9 cells cultivated over a range of dissolved oxygen concentrations (30 to 60% DO) in airlift and stirred tank bioreactors (Schlaeger & Schmid, unpublished results). Both, low-serum containing (1% FBS) or protein-free media IP301 and SF–1 (Schlaeger *et al.*, 1993) supplemented with lipids (in the form of fatty acid/sterol containing microemulsions or lipoprotein fractions) and Pluronic F–68 support cell growth to final densities of ca. 1 × 10E7 cells ml^{-1} without nutrient feeding. Hensler & Agathos (1994) found, contrary to the results obtained by Jain *et al.* (1991a,b), that Sf9 cells showed no difference with respect to specific cell growth rates, maximum cell densities (5 × 10E6 cells ml^{-1}) and cell viabilities, when cultivated in 0.25 1 stirred tank reactors using surface aeration over the whole range of DO levels from 5 to 100%.

Studies on the influence of dissolved oxygen on the growth of 2 different insect cell lines have recently been published. Deutschmann & Jäger (1994) reported optimal growth of Sf21 cells (the parental line to Sf9) at 70% DO using a 1.2 1 bioreactor equipped with a double-membrane stirrer for bubble-free aeration and medium perfusion. At 100% DO and unexpectedly also at 40% DO specific growth rates and maximum viable cell concentrations were adversely effected in batch experiments. At 40% air saturation maximum cell numbers were reduced more than threefold and population doubling times were increased ca. 50% compared to optimal conditions. Bombyx mori (Bm5) cell growth was evaluated by Zhang *et al.* (1994). Specific growth rates ($\mu = 0.46\ day^{-1}$) and maximum cell densities (ca. 4.3 × 10E6 cells ml^{-1}) were unaffected at DO levels between 20 and 60% air saturation. However, the maximum cell concentration was reduced to 3.6 × 10E6 cells ml^{-1} at 10% DO, which – as reasoned by Jain *et al.* (1991b) – could be due to the limited availability of oxygen for cellular functions.

Table 3. Bioreactor studies examining the effect of different dissolved oxygen concentrations on the product formation in insect cells

Cell line	DO setpoint (% air saturation)	Product expressed	References
Sf9	20, 40, 60, 80	polyhedra	Klöppinger *et al.*, 1990
Sf9	35, bioreactor (oxygen limitation)	epoxide hydrolase	Wang *et al.*, 1993
Sf9	10, 65, 110	half-antistasin (anticoagulant protein, inhibitor of Factor Xa)	Jain *et al.*, 1991a, b
Sf9	15, shake flask (oxygen excess)	β-galactosidase	Reuveny *et al.*, 1993
Sf9	10, 30, 50, spinner flask (oxygen limitation)	extracellular domain of human IFNγ receptor	Schmid *et al.*, 1994
Sf9	5, 10, 50, 100	β-galactosidase	Hensler & Agathos, 1994
BTI-Tn–5B1–4	15, 50	soluble human TNF p55 receptor	Schlaeger *et al.*, unpublished results
Sf9	5, 10, 50, 80, spinner flask (oxygen excess or limitation)	fusion protein of viral origin (accumulates in nuclei of infected cells)	Blanchard & Ferguson, 1992; Scott *et al.*, 1992
Bm5	30, 40	bacterial chloramphenicol acetyltransferase	Zhang *et al.*, 1994

Bm5 cells seems to be similar to Sf9 cells with regard to the observed broad optimum in dissolved oxygen concentration.

Although obtained with different culture media and bioreactor configurations, the sum of the above results seems to indicate that at least Sf9 and Bm5 cells can be grown over a wide range of DO concentrations extending from 20 to 65% DO at maximum specific growth rates and high cell densities. More extreme values in dissolved oxygen concentrations led to significantly reduced growth rates and cell concentrations except for the study communicated by Hensler & Agathos (1994).

Effect of dissolved oxygen concentration on recombinant protein and baculovirus production

Some groups also reported on the influence of different DO concentrations during the infection phase with wild-type or recombinant baculoviruses. Data eluding to the cell line under investigation, the dissolved oxygen during infection and the expressed protein are summarized in Table 3.

The effect of dissolved oxygen levels on the production of a truncated form of the anticoagulant and antimetastatic agent antistasin (H-ANS) was assessed in a silicon-tube gassed 8 1 bioreactor to exclude any potential effects of sparging (Jain *et al.*, 1991a,b). Cells were grown at 65% DO and then infected at different DO concentrations. As observed for Sf9 cell growth (see above), it was found that infection of the culture at 65% DO gave optimal H-ANS values, whereas DO levels of 10 and 110% resulted in decreased product yields and an almost 2-fold reduction of the specific productivity. H-ANS concentrations reached their maximum values at 80 hr post-infection (65% DO) and subsequently decreased, as measured by a Factor Xa inhibition assay.

In my group we performed similar experiments to evaluate the effects of different DO levels during infection on the expression of full length extracellular domains of human and mouse IFNγ receptors (Schmid *et al.*, 1994). In one experiment we used 2 identical 251 airlift bioreactors operated at a constant gas sparging rate of 0.05 VVM and a DO level of 50% during the growth phase of Sf9 cells. After identical batch growth, cultures were infected with the human recombinant virus preparation at a multiplicity of infection (MOI) of 1 pfu $cells^{-1}$. For one of the reactors the DO level was reduced to 10% air saturation ca. 15 min before infection. Data from ELISA determinations, functional binding assays, and electrophoretic analyses demonstrated a threefold increase in human IFNγ receptor concentrations when the infection process was carried out at the 10% compared to the 50% DO level. Multiple glycoforms of human (and mouse) soluble receptor(s) with apparent molar masses of 28 000 to 32 000 g mol^{-1} were observed for fermentation samples analyzed by SDS-PAGE under nonreducing conditions with subsequent ligand blotting or protein-staining. The pattern of identified isoforms varied as a function of infection time and DO level. Protein het-

erogeneity could be associated with either the unequal utilization of the five potential N-glycosylation sites or the linkage of different carbohydrate moieties to these sites or both (Fountoulakis *et al.*, 1991; Manneberg *et al.*, 1994). Taken together our data for the expression of human IFNγ receptor in Sf9 cells indicate that the highest expression levels (ca. 10 $\mu g\ ml^{-1}$ at 5 days post-infection) are obtained at 10 and 30% DO, whereas at 50% DO and at potentially oxygen limiting conditions (simulated by transferring cultures from bioreactors directly after infection into spinner flasks with various ratios of culture to vessel volume) tilers are drastically reduced. At day 6 (and later) of the post-infection period, ELISA and binding assay data indicate decreased concentrations of functionally active receptor protein, which may be a consequence of limited proteolytic degradation, changes in glycosylation pattern and/or other unidentified modifications.

Blanchard & Ferguson (1992) investigated the expression of a fusion protein of viral origin which accumulates in the nucleus of infected cells in a 3.5 1 stirred tank bioreactor using EX-CELL 401 serum-free medium. Sf9 cells were cultivated at 50% DO and at a viable cell density of 2.3 × 10E6 cells ml^{-1} infected with recombinant baculovirus at an MOI of 1 pfu $cell^{-1}$. In this study the DO level was then simultaneously with the virus addition reset to maintain the levels for the post-infection period at either 80, 50, 10 or 5% DO. The highest product concentrations were determined at 50% DO during infection (corresponds to 82% of the liter measured for the reference flask under oxygen excess). At 10 and 80% air saturation protein yields were reduced by 18 and 50%, respectively. At the lowest DO level only 5% of the maximum product concentration was obtained. It should be noted that the above values indicate protein titers after an infection period of 44 hr; no time-course data is presented. Using a recombinant baculovirus encoding for the same or a similar protein of interest, Scott *et al.* (1992) found no expression in an oxygen-limited spinner flask and a maximum product concentration ca. 50 hr post-infection in the bioreactor (50% DO). The group observed a decrease in cell viablity from greater 90 to 50% after 2 days of infection for the culture maintained at 50% DO, which resulted in the highest product tilers. This is in agreement with results by Schmid *et al.* (1994), who determined a lower remaining cell viability after 6 days of infection in the case of low (15%) dissolved oxygen concentration but higher IFNγ receptor concentrations. Jain *et al.* (1991b), however, did not observe an increased cell viability for 10% DO post-infection (compared to 65%) which was associated with 50% reduced H-ANS tilers. Culture viability as well as cell volume (Schmid *et al.*, 1994; Jain *et al.*, 1991b) may be a useful indicator to follow during the infection period for a given project However, a comparison of results from several groups is complicated by the use of various bioreactor configurations resulting in differenthydrodynamic environments during infection and by the use of dissimilar recombinant baculoviruses for infection (expression vectors and protein product itself).

Wang *et al.* (1993) found the expression of epoxide hydrolase from Sf9 cells increased by 200% when the dissolved oxygen was maintained at ca. 35% DO during the infection period compared to the oxygen-limited control. Reuveny *et al.* (1993) presented data on the effect of DO levels on recombinant β-galaclosidase production. Sf9 cells were propagated in a 5 1 stirred tank reactor at 65% DO using orifice sparging. After 4 days of infection cultures maintained at 15% air saturation in the bioreaclor yielded only 70% of product compared with cultures which were kept under conditions where oxygen was supposedly not limiting, i.e., shake flask cultures. However, when examining the time-course data for total β-galactosidase expression it seems as if the maximum concentration had been reached at day 4 post-infection in the shake flask culture, whereas tilers in the bioreactor were still increasing significantly from day 3 to day 4. This may indicate that maximum β-galactosidase concentrations were not yet achieved in this case and stresses the importance of evaluating the complete time-course of protein expression during the post-infection period.

Contrary to the above studies and similar to their experiments that investigated the effects of DO on Sf9 cell growth, Hensler & Agathos (1994) observed expression of β-galactosidase at identical levels over a wide range of dissolved oxygen concentrations between 5 and 100% air saturation.

For bacterial chloramphenicol acetyltransferase (CAT) production in Bombyx mori (Bm5) cells Zhang *et al.* (1994) found no difference in CAT yields for infection at either 30 or 40% DO. Trichoplusia ni (BTI-Tn–5Bl–4) cells were grown at 30% DO in 2 identical airlift bioreactors up to an infection cell density of 2.5 × 10E6 cells ml^{-1} by Schlaeger *et al.* (unpublished results). When the dissolved oxygen concentration was either adjusted to 15 or 50% air saturation during the infection period, product concentrations for soluble human TNF receptor p55 protein were determined to

be reduced by ca. 20% at the low DO level, whereby titers in the supernatant followed parallel time-courses. In a study that examined virus production in Sf9 cells at day 4 after infection with wild-type Autographa californica nuclear polyhedrosis virus, Klöppinger *et al.* (1990) reported that a dissolved oxygen concentration of 20% during infection reduced the yield of polyhedra per cell by more than 50% compared an oxygen concentration of 40 to 80%.

In summary, in all but one (Hensler & Agathos, 1994) of the investigations into the effect of DO levels on recombinant protein or polyhedra production significant differences in product yield were determined. From most publications it is not clear at what time exactly the dissolved oxygen level was changed from its growth phase value to the various post-infection values. It may be interesting to study the effect of DO more thoroughly, i.e., to adjust it to the desired levels some time before the addition of the baculovirus preparation, simultaneously with the virus addition, at the time of viral replication (15–24 hr post-infection) or later during the protein production phase. In any event, it is necessary to evaluate the complete time-course of product formation with respect to protein concentration and quality. Product quality is at least as important as total concentration because in the end it is the amount of intact biologically active product that determines the overall yield and productivity of any production process.

Effect of other physicochemical conditions

Temperature. Most insect cells can be cultivated over a temperature range of 25–30 °C (Agathos *et al.*, 1990); however, the optimal temperature during cell growth and infection for Sf9 cells is traditionally considered to be around 27–28 °C. In spinner flask studies Hild *et al.* (1992) achieved maximum cell densities and specific growth rates of 2.9–3.8 × 10E6 cells ml^{-1} and 0.67 day^{-1}, respectively, for Sf9 cells cultivated in TC100 medium with 5% FBS over a temperature range of 26–30 °C. Reuveny *et al.* (1993) found a temperature of 27 °C optimal for the growth of Sf9 cells resulting in the highest maximum cell concentrations and a specific growth rate of 0.67 day^{-1}. Already at 25 °C the specific growth rate was reduced by 30%. At 30 °C (while the specific growth actually was increased) an immediate and dramatic decrease in cell viability was observed after the maximum cell density was reached. This study appears to be the only one published where the effects of temperature on recombinant protein expression were examined. Exponentially growing cells cultivated under controlled conditions in 51 bioreactors (27 °C) were resuspended in fresh medium at 0.5 × 10E6 cells ml^{-1} and incubated in spinner flasks at different temperatures. Cells were infected at MOI of 3 with recombinant baculoviruses and the expression of β-galactosidase and human glucocerebrosidase was monitored both in the cell pellet and in the supernatant. The total expression levels at 27 °C were similar to those obtained at 22 and 25 °C; lower yields were obtained at 30 °C. An increase in temperature from 22 to 27 °C led to an earlier infection of cells, as indicated by earlier expression of proteins, and to an increase in the proportion of both products released into the medium. No analyses of protein quality (e.g., degradation or glycosylation) were performed. **pH-value.** Medium pH-values required for optimal in vitro growth of various insect cells range between pH 6 and 7 as given in the literature (Sohi, 1980; Hink, 1982; Kurtti & Munderloh, 1984). Hild *et al.* (1992) found maximum cell densities and specific growth rates of 3.1–3.8 × 10E6 cells ml^{-1} and 0.72 day^{-1}, respectively, for Sf9 growth over a range of pH-values between 6.2 and 6.4. In a recent study Zhang *et al.* (1994) reported on the effect of pH on cell growth for Bm5 cells in 1.5l bioreactors at a controlled DO of 40% air saturation. The highest specific growth rates (0.43–0.46 day^{-1}) and maximum cell densities (approaching 4.5 × 10E6 cells ml^{-1}) were obtained in the pH range from 6.1 to 6.3. At lower and higher pH-values increased lag times, reduced specific growth rates, and decreased maximum viable cell densities were observed. Similar optimal values of pH 6.0 to 6.25 and pH 6.2 to 6.8 were reported by Hink & Strauss (1980) for Trichoplusia ni (TN–368) cells and by Sohi (1980) for three lepidopteran cell lines, respectively. A pH-value of 6.2 is generally used for Sf9 cell growth in controlled bioreactors. The influence of medium pH-values on recombinant protein expression has possibly never been thoroughly investigated. Zhang *et al.* (1994, 1993) noted that recombinant chloramphenicol acetyltransferase (CAT) production in Bm5 cells was reduced by >50%, when the pH-value during infection was controlled at pH 6.5 instead of pH 6.3 (STR, 80 rpm, DO 40%, 28 °C). **Osmolality.** The same authors reported for Bombyx mori (Bm5) cells in flask experiments a maximum cell density at a medium osmolality of about 370 mosm kg^{-1}. Greater than 90% of the maximum cell density was achieved with a medium osmolality between 350 and 385 mosm kg^{-1}. In earlier studies reported values of optimal medium osmolalities for the growth of var-

ious insect cell lines vary between 250 and 450 mosm kg^{-1} (Sohi, 1980; Kurtti & Munderloh, 1984; Kurtti *et al.*, 1974; Kurtti *et al.*, 1975). Typical insect cell culture media are adjusted to an initial osmolality of 330–375 mosm kg^{-1} (Weiss *et al.*, 1981 and 1989; Schlaeger *et al.*, 1993; Wilkie *et al.*, 1980; Inlow *et al.*, 1989), whereas culture media for mammalian cells (hybridomas, CHO) usually have an osmolality of 280–320 mosm kg^{-1}. No studies have been reported on the influence of culture osmolality during the infection phase of insect cells.

Metabolic studies in batch and continuous cultures

Substrate and by-product metabolic quotients

Data on metabolic quotients for insect cells in the published literature is inconsistent. Hensler and Agathos (1994) determined that glucose and glutamine were consumed at 60–70% higher rates 24 hr after infection, at which time a maximum value in specific oxygen uptake rate was observed (see below). The specific glucose (qGluc) and glutamine (qGln) consumption rates were increased from 1.1 and 0.9 mmol/10E9 cells × d for uninfected cells to 1.8 and 1.5 mmol/10E9 cells × d for β-galactosidase-infected cells. Zhang *et al.* (1993) also found glucose uptake rates increased from 1.3 to 2.0 mmol/10E9 cells × d after viral infection, whereas Reuveny *et al.* (1992) found qGluc during the first 2 days after infection at same level or even reduced for either serum-containing or serum-free medium. Recent data by Wong *et al.* (1994) is in agreement with the latter results. Reductions in both qGluc and qGln were observed after infection with recombinant baculovirus, although a 30% increase in specific oxygen uptake rates was noted. The authors found some amino acid consumption rates (asparagine, arginine, glycine, threonine) elevated, but no indication that glucose or glutamine were responsible for the increased qO_2 after infection. They speculated that lipid catabolism is possibly contributing to the energy supply post-infection. Differences in nutrient consumption and by-product formation rates were observed by Reuveny *et al.* (1992), Kamen *et al.* (1991) and Bédard *et al.* (1993) as a function of culture medium. For a serum-free culture medium that contained glucose as the sole carbohydrate, Reuveny *et al.* (1992) calculated a 100% increase in qGluc compared to standard IPL–41 medium with 10% FBS that contained sucrose, maltose, glucose. Deutschmann & Jäger (1994) found the highest specific glucose uptake rate for Sf21 cells at 70% DO (1.5 mmol/10E9 cells × d). Under these conditions they observed the best growth characteristics with respect to specific growth rate and cell density. Uptake rates were reduced at lower specific growth rates (40 and 110% DO) to 0.8 and to 0.4 mmol/10E9 cells × d, respectively. No lactate formation was noted at 70 and 110% air saturation, however, the specific lactate formation rate was estimated at 3.6 mmol/10E9 cells × d at 40% DO. Schlaeger *et al.* (1995) compared qGluc and qGln during the exponential growth phase for Sf9 and BTI-Tn–5B1–4 cells cultivated in SF–1 medium. Specific rates for both nutrients were found to be higher for T. ni cells (qGluc 2.5 and qGln 1.1 mmol/10E9 cells × d) than for Sf9 cells (qGluc 1.9 and qGLN 0.6 mmol/10E9 cells × d).

Oxygen consumption rates

Volumetric oxygen consumption rates serve as one of the key design parameters for insect cell baculovirus production processes as they do for any other aerobic fermentation process. Over the past years several researchers and groups have reported specific oxygen uptake rates (qO_2) for insect cells during growth and subsequent infection with wild-type and recombinant baculoviruses (Table 4). From these values the volumetric oxygen demand can be estimated. The demand for insect cells may reach values as high as 100–150 mmol $l^{-1} \times d$. This compares to typical oxygen uptake rates of ca. 70 mmol $l^{-1} \times d$ for plant cells (Fowler, 1987) and of 100–2000 mmol $l^{-1} \times d$ for microorganisms (Enfors & Mattiasson, 1983).

Specific oxygen consumption rates determined for insect cells are similar to those obtained for mammalian cells (Spier & Griffiths, 1984; Fleischaker & Sinskey, 1981; Aunins & Henzler, 1993). Most research groups report an increasing specific oxygen requirement after infection with baculovirus preparations. The extent of increase varies significantly for the different studies. Variations may be due to the use of wild-type or recombinant baculoviruses, the differences in expression vectors and protein product, the multiplicity of infection (fraction of defective virus particles), the exact physiological state of cells at the time of infection or the physiological conditions during the infection period. Qualitatively the phenomenom of higher respiratory activity is attributed to increased metabolic rates (see above) that result from viral replication and virus-induced macromolecule biosynthesis.

Table 4. Specific oxygen consumption rates for different insect cells during growth and infection

Cell type	Cell line	Specific oxygen consumption rate (mmol/10E9 cells × d)		References
		Growth	Infection	
Insect	T. ni (TN–368)	8.6	17.3	Streett & Hink, 1978
	S. frugiperda (Sf9)	3.7 (exp. phase)	4.1	Maiorella *et al.*, 1988
	S. frugiperda (Sf9)	2.0–7.8 as function of DO	1.7–8.5	Jain *et al.*, 1991b
	S. frugiperda (Sf9)	9.2–11.4 (exp. phase)	9.5–3.7	Schmid *et al.*, 1994
	T. ni (BTI-Tn–5B1–4)	6.5–7.6 (exp. phase)	7.8–7.3	Schlaeger *et al.*, unpublished results
	S. frugiperda (Sf9)	4.8–6.5	7.7	Kamen *et al.*, 1991
	S. frugiperda (Sf9)	3.4–5.2	7.4–3.9	Schopf *et al.*, 1990
	S. frugiperda (Sf9)	4.8	–	Archambault *et al.*, 1994
	S. frugiperda (Sf9)	7.0	9.6	Scott *et al.*, 1992
	S. frugiperda (Sf9)	9.0–11.3 serum-free 5.3 serum-containing	–	Reuveny *et al.*, 1992 and 1993
	S. frugiperda (Sf21)	0.7–3.4 as function of DO	–	Deutschmann & Jäger, 1994
	S. frugiperda (Sf9)	4.7 (exp. phase) 1.9 (stat. phase)	3.9–2.7	King *et al.*, 1992
	S. frugiperda (Sf9)	5.3 (exp. phase)	6.9–4.0	Wong *et al.*, 1994
	S. frugiperda (Sf9)	7.9–8.4 serum-free 5.3 serum-containing	14.4–2.4	Hensler & Agathos, 1994; Hensler *et al.*, 1994
Mammalian	Various human cells, murine myelomas and hybridomas, recombinant CHO cell lines	0.8–13		Spier & Griffiths, 1984; Fleischaker & Sinskey, 1981; Aunins & Henzler, 1993

Streett & Hink (1978) measured an oxygen uptake rate of 8.6 mmol/10E9 cells × d for growing Trichoplusia ni TN–368 insect cells, doubling to 17.3 mmol/10E9 cells × d 14 hr post-infection with wild-type Autographa californica nuclear polyhedrosis virus. Other groups have not determined such a dramatic increase in oxygen consumption rates. Maiorella *et al.* (1988) found similar oxygen uptake rates during exponential growth and 21 hr post-infection at 3.7 mmol/10E9 cells × d and 4.1 mmol/10E9 cell × d, respectively. Sf9 cultures were infected at ca. $2.5 \times 10E6$ cells ml^{-1} and 20% air saturation with recombinant baculovirus encoding for human macrophage colony stimulating factor (M-CSF). The specific oxygen uptake rate during infection of Sf9 cultures with a recombinant virus encoding for a truncated form of antistasin was determined by Jain *et al.* (1991 b) at 3 different DO values (10, 65 and 110%). Experiments were performed in an 8 1 stirred tank bioreactor using bubble-free silicone tube gassing to exclude any potential deleterious effects due to sparging. The qO_2 at time of infection, i.e., of uninfected Sf9 cells, and over the whole infection period was significantly increased at the higher DO levels. Values at 65% DO were twice and values at 110% DO four times higher than those measured at 10% DO level. Specific rates remained relatively constant post-infection over 60 hr. Absolute values at 65% DO are comparable to the data reported by Maiorella *et al.* (1988). There was an increase of less than 10% in qO_2 with the maximum rate measured about 20 hr post-infection. Streett & Hink (1978), Schopf *et al.* (1990), Schmid *et al.* (1994), Wong *et al.* (1994) and Hensler & Agathos (1994) likewise observed a maximum qO_2 at 10–20 hr post-infection.

Schmid *et al.* (1994) combined on-line determinations of volumetric uptake rates with off-line hemacytometer determinations of viable cell concentrations to estimate the specific oxygen consumption rates for Sf9 cells during growth and infection with recombinant baculovirus encoding for soluble human IFNγ receptor. After growth at 50% dissolved oxygen with

an estimated qO_2 of 10 mmol/10E9 cells × d, the two identical 25 1 airlift bioreactors were maintained at 10 and 50% DO levels during the infection period. In both cases a transient maximum in specific oxygen uptake rates after infection was observed at ≤ 20 hr after the addition of recombinant virus (qO_2 increased by 10%, if conditions remained at 50% DO). This was then followed by a continuous decline in qO_2 values over a 5-day period to a level of about 50% of the pre-infection uptake rates. When the oxygen concentration was lowered to 10% DO, an immediate drop in qO_2 was seen followed by a similar decline phase as recorded for 50% DO during infection. The uptake rates always remained higher for the reactor controlled at the higher DO level. This is in agreement with the data obtained by Jain *et al.* (1991 b). As the Sf9 cell diameter increases from 14–16 μm during the exponential growth phase to 19–21 μm at days 2 to 4 post-infection (Schopf *et al.*, 1990; Schmid *et al.*, 1994; Jain *et al.*, 1991 b) a continuous decline in qO_2 values was determined when rates were expressed on a per cell volume basis.

Measurements of oxygen uptake rates for Trichoplusia ni (BTI-Tn–5Bl–4) cells (Schlaeger *et al.*, unpublished results) during exponential growth at 30% DO and 5.3 × 10E5 cells ml^{-1} gave values of 7.1 mmol/10E9 cells × d. When the viable cell concentrations reached 2 × 10E6 cells ml^{-1}, cultures were infected with recombinant baculovirus (soluble human TNF receptor p55 construct). Post-infection DO levels were controlled at 30%. At 24 and 47 hr after infection qO_2s remained practically unchanged at 7.8 and 7.3 mmol/10E9 cells × d, respectively. Culture characteristics seem to be similar to those of Sf9 cells. By on-line mass spectrometry Kamen *et al.* (1991) measured a qO_2 of ca. 4.8 mmol/10E9 cells x d during exponential growth of Sf9 cells in a helical ribbon bioreactor (Kamen *et al.*, 1992) using serum-free IPL–41 medium. Later in this batch experiment after cells had been diluted with fresh medium to adjust the cell concentration at about 1 × 10E6 cells ml^{-1} the authors estimated specific consumption rates of 6.5 mmol/10E9 cells × d prior to infection and of 7.7 mmol/10E9 cells ml^{-1} ca. 23 hr post-infection (i.e., a 15% increase).

Other groups have published greater increases in specific oxygen uptake rates. In another study where wild-type virus was used for infection of Sf21 cells, Weiss *et al.* (1982) reported a 25% increase in qO_2 after infection. When Schopf *et al.* (1990) determined off-line consumption rates of T-flask cultured Sf9 cells infected with wild-type and recombinant baculovirus encoding for β-galactosidase at multiplicities of infection (MOI) of 1 and 10 pfu $cell^{-1}$, they observed an increase in qO_2 from 3.4 mmol/10E9 cells × d to a maximum value of ca. 7.4 mmol/10E9 cells × d for recombinant β-galactosidase infected cells. These cells had a nearly 40% higher uptake rate than uninfected cells. For both MOIs the maximum respiratory activity occurred at 16 hr post-infection, followed by a sharp decline. Scott *et al.* (1992) reported increases of 40% in qO_2 from 7 to 9.6 mmol/10E9 cells × d for Sf9 cells infected with a recombinant virus encoding for the core and NS5 regions of hepatitis C virus. Specific oxygen uptake rates (7.9 mmol/10E9 cells × d) for β-galactosidase infected cells (Hensler & Agathos, 1994) increased almost twofold within 24 hr of infection, followed by a gradual decrease until, by 100 hr post-infection, cell respiratory activity had virtually stopped. An average qO_2 of 5.3 mmol/10E9 cells × d was estimated by Wong *et al.* (1994) for uninfected Sf9 cells during exponential growth in a 1 1 stirred tank bioreactor using serum-free medium (DO 50%). For infected cultures, the specific oxygen uptake rate increased immediately after addition of virus and a maximum of 1.3 times the value of uninfected cells was noted for all cultures between 8 and 30 hr post-infection, which coincides with the period at which most viral replication and the majority of DNA synthesis takes place. It was observed that the rate of rise in qO_2 decreased as the cell density at the time of infection increased, which meant that the later the infection, the later the maximum qO_2 was observed.

Deutschmann & Jäger (1994) found the qO_2 for Sf21 cells strongly related to cell growth. They calculated a specific oxygen uptake rate of 3.4 mmol/10E9 cells × d for optimal growth at 70% DO. At a DO concentration of 40% and a significantly reduced growth rate (μ = 0.39 day^{-1}), however, the qO_2 value was reduced to 0.7 mmol/10E9 cells × d. Hensler *et al.* (1994) and Hensler & Agathos (1994) like Reuveny *et al.* (1992) reported consistently increased oxygen consumption rates for uninfected Sf9 cells cultivated in serum-free media compared to the same cells grown in serum-containing media. This may have been the result of selecting a cell clone with altered characteristics or reflects a change in metabolic activity of cells in the serum-free environment.

Carbon dioxide production rates and respiration quotients

The respiration quotient (RQ) is a key metabolic parameter, that is independent of cell number or biomass

and can be measured on-line. It reflects the physiological state of cells and is needed to quantify main fluxes of cellular metabolism.

Recently, several publications have appeared in the literature that present data on specific carbon dioxide production rates (qCO_2) and respiration quotients (i.e., the ratio of the specific carbon dioxide production rate divided by the specific oxygen consumption rate) for uninfected and recombinant baculovirus-infected Sf9 cell cultures (Kamen *et al.*, 1991; Archambault *et al.*, 1994; Bedard *et al.*, 1994; Kamen & Tom, 1994). The authors report values of 2.6–7.4 mmol/10E9 cells × d for qCO_2s and values of 1.07 ± 0.06 for RQs in uninfected Sf9 cells cultures. Carbon dioxide production rates increased to 10.3 mmol/10E9 cells × d in virus-infected cultures with respiration quotients determined at 1.12 ± 0.03.

The partial pressure of carbon dioxide in the bioreactor off-gas was monitored by on-line mass spectrometry to estimate specific rates. Some data was obtained under uncontrolled or varying pH and dissolved oxygen conditions (Kamen *et al.*, 1991; Archambault *et al.*, 1994). All of the above studies were performed assuming negligible accumulation of carbon dioxide in the liquid phase, assuming fast carbon dioxide transfer at the gas/liquid interface (i.e., no liquid film resistance) and neglecting the presence of bicarbonate in the culture medium as well as the use of CO_2 for pH control, which will influence the partial pressure of CO_2 in the bioreactor off-gas during the first days of culture. In the light of recent experiments by Bonarius *et al.* (1995), who determined specific carbon dioxide production rates and respiration quotients for the growth of hybridoma cells in bicarbonate-buffered continuous culture (taking into account the above mentioned considerations), a more rigorous investigation into the changes of qCO_2s and RQs of insect cells during growth and infection seems to be indicated. Additionally, disturbances in pH caused by the pulse-wise addition of acid (or CO_2) and alkaline (Royce, 1992) may prove more significant in the case of batch cultures, where the goal would be to exploit the expected small differences in RQ values for control purposes. On-line measurements of respiration quotients of sufficient accuracy may contribute to the process optimization of insect as well as mammalian cell cultures.

Conclusions and outlook

The complete strategy for maximizing the yield of recombinant proteins from insect cell culture must include an optimization of the culture conditions during the growth phase as well as during the subsequent infection phase. The growth of host cells like Spodoptera frugiperda (Sf9 and Sf21) and Trichoplusia ni (BTI-Tn–5Bl–4) to cell densities of ca. 10 × 10E6 cells ml^{-1} in batch cultures has so far been achieved. Already today some groups have reported even higher viable cell concentrations ($>10 \times 10E6$ cells ml^{-1}) using nutrient feeding strategies. There will be further improvements in this area. However, probably more important will be the characterization of the optimal physiological state that the cells – at high densities – have to be in at the time of infection so as to maintain the same (or reach even higher) specific productivities than in low-density infections.

The effects of culture conditions (pH, θ, pCO_2, DO, nutrients) during the infection phase on product yield and quality need to be more thoroughly investigated. The cells may potentially have different requirements during the viral replication phase and the following protein production phase. Hardly anything seems to be known about protease activities in Sf9 insect cells (Jäger *et al.*, 1992). Other insect host cells that are currently investigated (Betenbaugh *et al.*, 1991; Davis *et al.*, 1993; Wickham *et al.*, 1992) may have improved characteristics in the respect.

References

Agathos SN, Jeong Y-H & Venkat K (1990) Growth kinetics of free and immobilized insect cell cultures. Annals of the New York Academy of Sciences 589: 372–398.

Archambault J, Robert J & Tom L (1994) Culture of immobilized insect cells. Bioprocess Engineering, 11, 189–197.

Aunins JG & Henzler H-J (1993) Aeration in cell culture bioreactors. In: Rehm H-J & Reed G (Series Eds.), Biotechnology: a multivolume comprehensive treatise, Vol. 3, 2nd Edition (pp. 219–281). Weinheim: VCH.

Barkhem T, Carlsson B, Danielsson A, Norinder U, Frieberg H & Öhman L (1992) Production in a 100 l stirred tank reactor of functional full length human thyroid receptor betal in Sf9 insect cells using a recombinant baculovirus. In: Vlak JM, Schlaeger E-J & Bernard AR (eds), Baculovirus and recombinant protein production processes (pp. 235–246), Basel: Editiones Roche.

Bédard C, Jolicoeur M, Jardin B, Tom R, Perret S & Kamen AA (1994) Insect cell density in bioreactor cultures can be estimated from on-line measurements of optical density. Biotechnology Techniques 8: 605–610.

Bédard C, Tom R & Kamen AA (1993) Growth, nutrient consumption, and end-product accumulation in Sf–9 and BTI-EAA insect cell cultures: Insights into growth limitation and metabolism. Biotechnology Progress 9: 615–624.

Betenbaugh MJ, Balog L & Lee P-S (1991) Production of recombinant proteins by baculovirus-infected gypsy moth cells. Biotechnology Progress 7: 462–467.

Blanchard JH & Ferguson CHR (1992) The effect of different levels of dissolved oxygen on recombinant protein production in Sf–9 cells. In: Vlak JM, Schlaeger E-J & Bernard AR (eds), Baculovirus and recombinant protein production processes (pp. 247–254). Basel: Editiones Roche.

Bonarius HPJ, Gooijer de CD, Tramper J & Schmid G (1995) Determination of the respiration quotient in mammalian cell culture in bicarbonate buffered media. Biotechnology and Bioengineering 45: 524–535.

Caron AW, Archambault J & Massie B (1990) High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. Biotechnology and Bioengineering 36: 1133–1140.

Caron AW, Tom RL, Kamen AA & Massie B (1994) Baculovirus expression system scaleup by perfusion of high-density Sf–9 cell cultures. Biotechnology and Bioengineering 43: 881–891.

Chung IS, Taticek RA & Shuler ML (1993) Production of human alkaline phosphatase, a secreted, glycosylated protein, from a baculovirus expression system and the attachment-dependent cell line Trichoplusia ni BTI-Tn–5Bl–4 using a split-flow, air-lift bioreactor. Biotechnology Progress 9: 675–678.

Davis TR, Wickham TJ, McKenna KA, Granados RR, Shuler ML & Wood HA (1993) Comparative recombinant protein production of eight insect cell lines. In vitro Cellular and Developmental Biology 29A: 388–390.

Deutschmann SM & Jäger V (1994) Optimization of the growth conditions of Sf21 insect cells for high-density perfusion culture in stirred-tank bioreactors. Enzyme Microbial Technology 16: 506–512.

Enfors SO & Mattiasson B (1983) Oxygenation of processes involving immobilized cells. In: B Mattiasson (ed.), Immobilized cells and organelles, Vol. 2 (pp. 41–59). Boca Raton: CRC Press.

Fertig G, Rahn HP, Angermann A, Klöppinger M & Miltenburger HG (1993) Biotechnological aspects of the production of human pro-kallikrein using the AcNPV-baculovirus-expression system. Cytotechnology 11:67–75.

Fleischaker RJ & Sinskey AJ (1981) Oxygen demand and supply in cell culture. European Journal of Applied Microbiology and Biotechnology 12: 193–197.

Fountoulakis M, Schlaeger E-J, Gentz R, Juranville J-F, Manneberg M, Ozmen L & Garotta G (1991) Purification and biochemical characterization of a soluble mouse interferon gamma receptor produced in insect cells. European Journal of Biochemistry 198: 441–450.

Fowler MW (1987) Products from plant cells. In: Bu'Lock JD & Kristiansen (eds), Basic biotechnology (pp. 525–544). London: Academic Press.

Guillaume JM, Soria HM, Couteault N, Hurwitz DR, Multon MC & Crespo A (1992) Insect cell fermentation scale-up for recombinant protein production. In: Vlak JM, Schlaeger E-J & Bernard AR (eds), Baculovirus and recombinant protein production processes (pp. 255–261). Basel: Editiones Roche.

Hensler WT & Agathos SN (1994) Evaluation of monitoring approaches and effects of culture conditions on recombinant protein production in baculovirus-infected insect cells. Cytotechnology 15: 177–186.

Hensler WT, Singh V & Agathos SN (1994) Production of β-galactosidase in Sf9 insect cells in serum-containing and serum-free medium. Annals of the New York Academy of Sciences: 745: 149–166.

Hild HM, Emery AN & Al-Rubeai M (1992) The effect of pH, temperature, serum concentration and media composition on the growth of insect cells. In: Vlak JM, Schlaeger E-J & Bernard AR (eds), Baculovirus and recombinant protein production processes (pp. 316–321). Basel: Editiones Roche.

Hink WF & Strauss EM (1980) Semi-continuous culture of the TN–368 cell line in fermentors with virus production in harvested cells. In: Kurstak E, Maramorosch K & Dübendorfer A (eds), Invertebrate systems *in vitro* (pp. 27–33). Amsterdam: Elsevier/North-Holland Biomedical Press.

Hink WF (1982) Production of Autographa californica nuclear polyhedrosis virus in cells from large-scale suspension cultures. In: Kurstak E (ed), Microbial and viral pesticides (pp. 493–506). New York: Marcel Dekker.

Inlow D, Shauger A & Maiorella B (1989) Insect cell culture and baculovirus propagation in protein-free medium. Journal of Tissue Culture Methods 12: 13–16.

Jain D, Ramasubramanyan K, Gould S, Lenny A, Candelore M, Tota M, Strader C, Alves K, Cuca G, Tung JS, Hunt G, Junker B, Buckland BC & Silberklang M (1991a) Large-scale recombinant protein production using the insect cell-baculovirus expression vector system: Antistasin and beta-adrenergic receptor. In: Spier RE, Griffiths JB & Meignier B (eds), Production of Biologicals from Animal Cells in Culture (pp. 345–351). Oxford: Butterworth-Heinemann.

Jain D, Ramasubramanyan K, Gould S, Seamans C, Wang S, Lenny A & Silberklang M (1991b) Production of antistasin using the baculovirus expression system. In: Hatch RT, Goochee C, Moreira A & Alroy Y (eds), ACS Symposium Series No. 477 (pp. 97–110). American Chemical Society.

Jäger V, Grabenhorst E, Kobold A, Deutschmann SM & Conradt HS (1992) High density perfusion culture of insect cells for the production of recombinant proteins. In: Vlak JM, Schlaeger E-J & Bernard AR (eds), Baculovirus and recombinant protein production processes (pp. 274–284). Basel: Editiones Roche.

Junker BH, Hunt G, Burgess B, Aunins J & Buckland BC (1994) Modified microbial fermenter performance in animal cell culture and its implications for flexible fermenter design. Biotechnology Progress 11:57–63.

Kamen AA, Chavarie C, Andre G & Archambault J (1992) Design parameters and performance of a surface baffled helical ribbon impeller bioreactor for the culture of shear sensitive cells. Chemical Engineering Science 47: 2375–2380.

Kamen A A & Tom R (1994) Mass spectroscopy determination of insect cell respiration rates in culture and production processes. In: Spier RE, Griffiths JB & Berthold W (eds), Animal Cell Technology: Products of Today, Prospects for Tomorrow (pp. 345–350). Oxford: Butterworth-Heinemann.

Kamen AA, Tom RL, Caron AW, Chavarie C, Massie B & Archambault J (1991) Culture of insect cells in a helical ribbon impeller bioreactor. Biotechnology and Bioengineering 38: 619–628.

King GA, Daugulis AJ, Faulkner P & Goosen MFA (1992) Recombinant β-galactosidase production in serum-free medium by insect cells in a 14-L airlift bioreactor. Biotechnology Progress 8: 567–571.

King GA, Daugulis AJ, Goosen MFA, Faulkner P & Bayly D (1989) Alginate concentration: a key factor in growth of temperature-sensitive baculovirus-infected insect cells in microcapsules. Biotechnology and Bioengineering 34: 1085–1091.

Klöppinger M, Fertig G, Fraune E & Miltenburger HG (1990) Multistage production of Autographa californica nuclear polyhedrosis virus in insect cell cultures. Cytotechnology 4: 271–278.

Kompier R, Kislev N, Segal I & Kadouri A (1991) Use of a stationary bed reactor and serum-free medium for the production of recombinant proteins in insect cells. Enzyme Microbial Technology 13: 822–827.

Kurtti TJ, Chaudhary SPS & Brooks MA (1974) Influence of physical factors on the growth of insect cells *in vitro* I. Effect of osmotic pressure on growth rate of a moth cell line. In vitro Cellular and Developmental Biology 10: 149–156.

Kurtti TJ, Chaudhary SPS & Brooks MA (1975) Influence of physical factors on the growth of insect cells *in vitro* II. Sodium and potassium as osmotic pressure regulators of moth cell growth. In vitro Cellular and Developmental Biology 11: 274–285.

Kurtti TJ & Munderloh UG (1984) Mosquito cell culture. Advances in Cell Culture 3: 259–302.

Lazar A, Silberstein L, Reuveny S & Mizrahi A (1987) Microcarriers as a culturing system of insect cells and insect viruses. Developments in Biological Standardization 66: 315–323.

Lazarte JE, Tosi P-F & Nicolau C (1992) Optimization of the production of full-length rCD4 in baculovirus-infected Sf9 cells. Biotechnology and Bioengineering 40: 214–217.

Maiorella B, Inlow D, Shauger A & Harano D (1988) Large-scale insect cell-culture for recombinant protein production. Bio/Technology 6: 1406–1410.

Manneberg M, Friedlein A, Kurth H, Lahm H-W & Fountoulakis M (1994) Structural analysis and localization of the carbohydrate moieties of a soluble human interferon gamma receptor produced in baculovirus-infected insect cells. Protein Science 3: 30–38.

Murhammer DW & Goochee CF (1988) Scaleup of insect cell cultures: protective effects of pluronic F–68. Bio/Technology 6: 1411–1418.

Nguyen B, Jarnagin K, Williams S, Chan H & Barnett J (1993) Fed-batch culture of insect cells: a method to increase the yield of recombinant human nerve growth factor (rhNGF) in the baculovirus expression system. Journal of Biotechnology 31: 205–217.

Power JF, Greenfield PF, Nielsen L & Reid S (1992) Modelling the growth and protein production by insect cells following infection by a recombinant baculovirus in suspension culture. Cytotechnology 9: 149–155.

Power JF, Reid S, Radford KM, Greenfield PF & Nielsen LK (1994) Modeling and optimization of the baculovirus expression vector system in batch suspension culture. Biotechnology and Bioengineering 44: 710–719.

Reuveny S, Kemp CW, Eppstein L & Shiloach J (1992) Carbohydrate metabolism in insect cell cultures during cell growth and recombinant protein production. Annals of the New York Academy of Sciences 655: 230–237.

Reuveny S, Kim YJ, Kemp CW & Shiloach J (1993) Effect of temperature and oxygen on cell growth and recombinant protein production in insect cell cultures. Applied Microbiology and Biotechnology 38: 619–623.

Royce PN (1992) Effect of changes in the pH and carbon dioxide evolution rate on the measured respiratory quotient of fermentations. Biotechnology and Bioengineering 40: 1129–1138.

Schlaeger E-J, Foggetta M, Vonach JM & Christensen K (1993) SF–1, a low cost culture medium for the production of recombinant proteins in baculovirus infected insect cells. Biotechnology Letters 7: 183–188.

Schlaeger E-J, Loetscher H & Gentz R (1992) Fermentation scale-up: production of soluble human TNF receptors. In: Vlak JM, Schlaeger E-J & Bernard AR (eds), Baculovirus and recombinant protein production processes (pp. 201–208). Basel: Editiones Roche.

Schlaeger E-J, Stricker J, Wippler J & Foggetta M (1995) Investigations of high cell density baculovirus infection using Sf9 and High Five insect cell lines in the low-cost SF-1 medium. In: Beuvery EC, Griffiths JB & Zeijlemaker WP (eds), Animal Cell Technology: Developments Towards the 21st Century (pp. 313–315). Dordrecht: Kluwer Academic Publishers.

Schmid G, Wild N, Fountoulakis M, Gallati H, Gentz R, Ozmen L & Garotta G (1994) Production and characterization of soluble mouse and human interferon-γ receptors (IFNγ-R) from insect cells. In: Spier RE, Griffiths JB & Berthold W (eds), Animal Cell Technology: Products of Today, Prospects for Tomorrow (pp. 625–631). Oxford: Butterworth-Heinemann.

Schopf B, Howaldt MW & Bailey JE (1990) DNA distribution and respiratory activity of Spodoptera frugiperda populations infected with wild-type and recombinant Autographa californica nuclear polyhedrosis virus. Journal of Biotechnology 15: 169–186.

Scott RI, Blanchard JH & Ferguson CHR (1992) Effects of oxygen on recombinant protein production by suspension cultures of Spodoptera frugiperda (Sf–9) insect cells. Enzyme Microbial Technology 14: 798–804.

Sohi SS (1980) The effect of pH and osmotic pressure on the growth and survival of three lepidopteran cell lines. In: Kurstak E, Maramorosch K & Dübendorfer A (eds). Invertebrate systems *in vitro* (pp. 35–43). Amsterdam: Elsevier/North-Holland Biomedical Press.

Spier RE & Griffiths JB (1984) An examination of the data and concepts germane to the oxygenation of cultured animal cells. Developments in Biological Standardizati on 55: 81–92.

Streett DA & Hink WF (1978) Oxygen consumption of Trichoplusia ni (TN–368) insect cell line infected with Autographa californica nuclear polyhedrosis virus. Journal of Invertebrate Pathology 32: 112–113.

Wang M-Y, Kwong S & Bentley WE (1993) Effects of oxygen/glucose/glutamine feeding on insect cell baculovirus protein expression: a study on epoxide hydrolase production. Biotechnology Progress 9: 355–361.

Weiss SA, Belisle BW, DeGiovanni A, Godwin G, Kohler J & Summers MD (1989) Insect cells as substrates for biologicals. Developments in Biological Standardization 70: 271–279.

Weiss SA, Orr T, Smith GC, Kalter SS, Vaughn JL & Dougherty EM (1982) Quantitative measurement of oxygen consumption in insect cell culture infected with polyhedrosis virus. Biotechnology and Bioengineering 24: 1145–1154.

Weiss SA, Smith GC, Kalter SS & Vaughn JL (1981) Improved method for the production of insect cell cultures in large volume. In vitro Cellular and Developmental Biology 17: 495–502.

Wickham TJ, Davis T, Granados RR, Shuler ML & Wood HA (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnology Progress 8: 391–396.

Wickham TJ & Nemerow GR (1993) Optimization of growth methods and recombinant protein production in BTI-Tn–5Bl–4 insect cells using the baculovirus expression system. Biotechnology Progress 9: 25–30.

Wilkie GEI, Stockdale H & Pirt SV (1980) Chemically-defined media for production of insect cells and viruses *in vitro.* Developments in Biological Standardization 46: 29–37.

Wong TKK, Nielsen LK, Greenfield PF & Reid S (1994) Relationship between oxygen uptake rate and time of infection of Sf9 insect cells infected with a recombinant baculovirus. Cytotechnology 15: 157–167.

Wu J, King GA, Daugulis AJ, Faulkner P & Goosen MFA (1992) Recombinant protein production in insect cell cultures infected with a temperature-sensitive baculovirus. Cytotechnology 9: 141–147.

Zhang J, Kalogerakis N & Behie LA (1994) Optimization of the physiochemical parameters for the culture of Bombyx mori insect cells used in recombinant protein production. Journal of Biotechnology 33: 249–258.

Zhang J, Kalogerakis N, Behie LA & Iatrou K (1993) A two-stage bioreactor system for the production of recombinant proteins using a genetically engineered baculovirus/insect cell system. Biotechnology and Bioengineering 42: 357–366.

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Medium design for insect cell culture

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Introduction

Interest in insect cell culture technology has increased tremendously during the last two decades (Weiss *et al*., 1981; Grace, 1982; Smith *et al*., 1983; Summers & Smith, 1987; Luckow, 1991). Promising areas of application are the production of insect pathogenic viruses as insecticides for agricultural pest control and the production of recombinant proteins using the baculovirus expression system for biological research and human health care (Miltenburger & Krieg, 1984; Weiss & Vaughn, 1986; Wood & Granados, 1991; Weiss *et al*., 1992; Luckow, 1993). Many of the nuclear polyhydrosis viruses (NPV) which cause diseases in a number of insect species, are presently produced in insect cells. The majority of NPV's have been isolated from Lepidoptera (Miltenburger & Krieg, 1984; Vlak, 1992). The prototype of the baculovirus which is still commonly used, the Autographa California multiple nuclear polyhydrosis virus (AcMNPV), was originally isolated from the alfalfa looper, Autographa californica. Insect cell culture technology has become an important part of modern biotechnology and is increasingly used on large production scale. Consequently, the question of the availability of suitable insect cell culture media, especially for suspension culture, has become very important (Maiorella *et al*., 1988; Weiss *et al*., 1992). Because the large scale production of baculoviruses (OV's), or recombinant proteins after virus infection, can be run only as a batch process, the cultivation of insect cells should be effective and economical (Weiss *et al*., 1992).

Improved media are required which strongly support cell growth and the high yield production of baculoviruses, as well as the expression of recombinant proteins, at a competitive price (Maiorella *et al*., 1988; Wu *et al*., 1989). Animal serum, which is the supernatant of clotted blood, contains growth factors essential to mammalian cell growth. Fetal calf serum (FCS), also named fetal bovine serum (FBS), has been found to be particulary suitable with respect to its growth promoting properties in vertebrate cell culture. Insect blood, or hemolymph, is the in vivo medium for insect cells organized in tissues and organelles. Hemolymph circulates in an open system and simply bathes all tissues (Law & Wells, 1989). The nutrient requirements of insects are generally similar to those of vertebrates, with the exception that their diet must contain a source of cholesterol. Insects have no capacity for steroidogenesis (Law & Wells, 1989). Cholesterol is used for the formation of cell membranes and for the synthesis of the important steroid hormone 20-hydroxy-ecdysone (Law & Wells, 1989). A characteristic of insect blood is the high level of free amino acids (Wyatt, 1961; Grace, 1962). The total amino acids concentration in most insect hemolymphs varies from 300 mg to 2100 mg ml^{-1} due to the high osmotic pressure. Most insect cell culture media, therefore contain high levels of amino acids (Grace, 1962; Gardiner & Stockdale, 1975; Weiss *et al*., 1981). Furthermore, insect blood contains an unusually high level of free organic acids such as citrate, α-ketoglutarate, malate, fumarate, succinate, oxolate, and pyruvate; concentrations range from 0.1 to 25–35 mmoles per insect (Wyatt, 1961; Grace, 1962; Vaughn, 1968). The Krebs cycle intermediates are good chelating agents and therefore play an important role in the cationic balance of the hemolymph. However, their role in cell cul-

ture is unknown. They are probably of no importance (see below). Some media having reduced amounts, or none of these components, still support insect cell growth (Gardiner & Stockdale, 1975).

The general principles and methods for the culture of invertebrate cells are the same as those for vertebrate cells, the major difference being in the nature of the medium used. Based on the analysis of the chemical components in insect hemolymph the insect cell culture medium differs in several respects from the vertebrate medium used for the culture of mammalian cells (Vaughn, 1968): 1. The medium is more acidic, ranging in pH from 6.2–6.9, as observed in insect tissue fluids (Grace, 1962). The media are buffered with sodium phosphate, no CO_2 being required for insect cell culture. The media are clear to yellow due to the supplementation of protein hydrolysates. The pH indicator is omitted. 2. The osmotic pressure varies significantly from that in vertebrate blood, being more than twice as high (Vaughn, 1968). Commonly used insect cell culture media exhibit osmotic pressures of 340–390 mOsmol kg^{-1} compared to 290–330 mOsmol kg^{-1} for vertebrate media. Variations of ± 50 mOsmol kg^{-1} do not have a significant effect on cell growth properties (Schlaeger *et al.*, 1992A). 3. The ratio of the Na^+/K^+ ions differs in the base medium due to the different groups among the insects, although the general composition of the inorganic salt solutions resemble each other. Seawater (marine) invertebrates have a ratio of 1, the more specialized Lepidoptera exhibit a Na^+/K^+ ratio of less than 1. In more primitive insects, on the other hand, the ratio is greater than 1 (Vaughn, 1968). How important this ratio in vitro really is still has to be shown.

Besides these differences, culture media which support the growth and maintenance of insect cells are very similar to those used for mammalian cells. It should be mentioned that it was possible to adapt certain insect cell lines to grow in vertebrate media, as shown with a Tick cell line, which could be cultured in Dulbecco modified Eagle medium (Yunker *et al.*, 1981). Before medium development or modification of a given medium begins, it is important to define the demands of the user. Only a simplified medium is needed to grow and maintain the cells in culture. However, to fullfil requirements such as achieving a high cell densitiy with a reasonable population doubling time (PDT = 20–24 h) and high productivity of baculovirus as well as recombinant proteins the medium development has to be adapted to the specific metabolic needs of the cells. In Spodoptera frugiperda cell lines (Sf9 and Sf21), for example, the final consumption of amino acids is slightly different to that in BTI-TN–5B1–4 (Trichoplusia ni) cells which have an extremely high requirement for asparagine (and oxygen) (Schlaeger *et al.*, 1996).

The excess metabolism of glutamine and glucose, two essential nutrients in mammalian cell culture, which denotes incomplete oxidation, results in an excess production of ammonium and lactate respectively in a batch culture (Ljunggren & Häggström, 1992). The accumulation of these metabolic byproducts is often inhibitory (Glacken *et al.*, 1986; Butler & Jenkins, 1989; Hassell & Butler, 1990). However high concentrations of glutamine and glucose in insect cell culture do not result in critical concentrations of these metabolites at the end of the growth phase (Bédard *et al.*, 1992; Schlaeger *et al.*, 1992B). Detoxification of these metabolites in insect cells obviously follows a different pathway (Bédard *et al.*, 1992). Therefore, high concentrations of glucose and glutamine can be used in insect media to support high cell density growth (see later).

In this review, development of insect cell culture media particularly for cell growth, baculovirus propagation and recombinant protein production, are described for cell lines derived from the invertebrate type Lepidoptera. Two species of lepidopterous insects, the fall armyworm Spodoptera frugiperda (Vaughn *et al.*, 1977) and the cabbage looper Trichoplusia ni (Hink, 1970) were generally used. The established cell lines (Sf21, Sf9 and BTI-TN–5B1–4, also called High Five™) are susceptible to infection by Autographa californica nuclear polyhydrosis virus. This overview of the commonly used media does not consider the specific attention one has to give to isolating new cell lines from different insects. However, many cell lines, derived from other insect species, can be adapted to grow in the culture medium described here. Small modifications might have to be introduced to accommodate the specific requirements of certain cell lines.

Development of conventional media

The first significant breakthrough in the development of a useful insect cell culture medium was made by Wyatt and co-workers in 1956. This pioneering work was based on a chemical analysis of the hemolymph of the silkworm Bombyx mori. Wyatt formulated a synthetic medium containing high concentrations of amino acids, organic acids, inorganic salts and several sug-

ars supplemented with heat-treated hemolymph. Heat treatment of the insect fluid was required to destroy the activity of the enzyme polyphenol oxidase which forms toxic quinones in the presence of air (Wyatt, 1961). Wyatt's medium was then further improved by Grace, who added 9 water soluble B-complex vitamins, and altered the physiological characteristics of the medium. With the help of Grace (Antheraea) insect cell medium, the first invertebrate cell line from the ovarian tissue of the eucalyptus tree moth Antheraea eucalypti was established (Grace, 1962). Grace's modified medium, which still requires the supplementation of heat-treated hemolymph, was used for culturing cells derived from Lepidoptera. Grace's original medium has been modified several times (Hink, 1970; Gardiner & Stockdale, 1975; Goodwin & Adams, 1978).

The next step forward in the development of suitable invertebrate cell culture media came from vertebrate cell culture. It was known at that time that a culture medium, supplemented with protein hydrolysates, supported mammalian cell growth and maintainance of many established cell lines (Ginsberg *et al.* 1955; Amborski & Moskowitz, 1968; Hsueh & Moskowitz, 1972). Furthermore, addition of protein lysates could be substituted for serum, to a certain extent, depending on the cell line (Zoon *et al.*, 1979). Enzymatic protein hydrolysates of animal tissues, or microbial biomass, represent an inexpensive source of amino acids, and (oligo-) peptides consisting of hydrophilic and hydrophobic amino acids which can be associated with some essential lipids (see below). In addition, other fractions of known and unknown, low molecular weight compounds were described as functioning as growth promotors (Amborski & Moskowitz, 1968; Hsueh & Moskowitz, 1972; Yamane & Murakami, 1973). Similar growth supporting effects in the presence of digested extract, have also been observed in insect cell cultures (Jones & Cunningham, 1961, Mitsuhashi & Maramorosch, 1964; Hink, 1970). The addition of protein hydrolysates (lactalbumin, tryptose phosphate broth, casein, yeast extract or also called yeastolate) to insect cell culture media is extremely useful, especially at higher concentrations than would be tolerated by mammalian cell lines. Mitsuhashi and Maramorosch used protein hydrolysates in their MM insect culture medium developed in 1964. In this simplified medium, with a standard salt solution, lactalbumin and yeastolate supplemented the use of single amino acids and purified vitamins respectively.

The most successful modification of Grace's original medium, was carried out by Hink in 1970. He added lactalbumin, 3.3 g l^{-1}, together with yeastolate, 3.3 g l^{-1}, as a sources for vitamins (B-complex), to Grace's base medium, and replaced insect hemolymph with 10% of heat-inactivated fetal bovine serum. Although vertebrate serum probably did not fullfil all the specific lipid requirements of insect cells (Goodwin, 1991), the use of animal serum was a significant improvement at that time. Serum was probably cheaper and it was available in large quantities. In contrast to Mitsuhashi's MM medium, Hink's TNM-MH medium contained all of Grace's amino acids and vitamins and was originally developed for the isolation of theTN–368 cell line from cabbage looper Trichoplusia ni (T. ni). Another modification of Grace's original medium is TC–100, originally named BML-TC/10 (Gardiner & Stockdale, 1975). TC–100 was optimized for the growth of Autographa californica nuclear polyhedrosis virsus in Sf-cells by the omission of organic acids, addition of glucose as the only hexose source, and its supplementation with tryptose extract (2.6 g l^{-1}) and fetal bovine serum. TC–100 is a simplified insect cell culture medium for laboratory use, but not for large scale production (Cameron *et al.*, 1989).

An important step forward was made with the development of the more complex IPL–41 medium at the USDA Insect Pathology Lab by Weiss et al 1981. IPL–41, which is a modification of Goodwin's original IPL formulation (Goodwin, 1975), was originally developed to improve the insect cell culture process on a large scale, for the efficient production of baculoviruses. Compared to Grace's medium, the concentration of many amino acids, vitamins and other medium components are significantly increased. The basic formulation of the three most commonly used insect cell culture media, Grace (TNM-FH), TC–100, and IPL–41, are listed in Table 1. Addition of glucose (2–4 g l^{-1} in TC–100) and protein hydrolysates (lactalbumin, tryptose phosphate broth, yeastolate in TC 100 and IPL–41) would make these media more powerful. In conclusion; in these more conventional insect cell culture media formulations, which mimic the physiological properties of the insect fluids, the addition of heat inactivated hemolymph was replaced by vertebrate serum. Furthermore cell growth and virus propagation were supported by the addition of protein hydrolysates.

It should be noted here that reproducible preparations of a given conventional culture medium from the individual components is labor intensive and propably not really cost effective, if only lab scale volumes are needed. However, if specialized media are required,

Table 1. Composition of conventional insect cell culture media ($mg\,l^{-1}$)[a]

Components	Grace's (supplemented)	IPL–41	TC–100
	(liquid)		
Inorganic Salts			
$CaCl_2$	750.00	500.00	980.00
$CoCl_2 \cdot 6H_2O$	–	0.05	–
$CuCl_2 \cdot 2H_2O$	–	0.20	–
$FeSO_4 \cdot 7H_2O$	–	0.55	–
KCl	4100.00	1200.00	2900.00
$MgCl_2 \cdot 6H_2O$	2280.00	–	–
$MgCl_2$	–	–	1070.00
$MgSO_4 \cdot 7H_2O$	2780.00	–	–
$MgSO_4$	–	918.00	1370.00
$MnCl_2 \cdot 4H_2O$	–	0.02	–
NaCl	–	2850.00	–
$NaHCO_3$	350.00	350.00	350.00
$NaH_2PO_4 \cdot H_2O$	1013.00	1160.00	–
$NaH_2PO_4 \cdot 4H_2O$	–	–	1100.00
$(NH_4)(Mo_7O_{24} \cdot 14H_2O)$	–	0.04	–
$ZnCl_2$	–	0.04	–
Sugars, Other			
D(-)-Fructose	400.00	–	–
Fumaric acid, free acid	55.00	4.40	–
D(+)-Glucose	700.00	2500.00	1000.00
a-Ketoglutaric acid	370.00	29.60	–
L(-)Malic acid	670.00	53.60	–
Maltose	–	1000.00	–
Succinic acid	60.00	4.80	–
Sucrose	26680.00	1650.00	–
Amino acids			
β-Alanine	200.00	300.00	–
L-Alanine	225.00	–	225.00
L-Arginine			550.00
L-Arginine HCl	700.00	800.00	–
L-Asparagine	350.00	1300.00	350.00
L-Aspartatic acid	350.00	1300.00	350.00
L-Cystine	22.00	–	–
L-Cystine 2Na	–	130.35	–
L-Glutaminic acid	600.00	1500.00	600.00
L-Glutamine	600.00	1000.00	600.00
Glycine, free base	650.00	200.00	650.00
L-Histidine	2500.00	200.00	3400.00
L-Isoleucine	50.00	750.00	50.00
L-leucine	75.00	250.00	75.00
L-Lysine. HCl	625.00	700.00	630.00
L-Methionine	50.00	1000.00	50.00
Amino acids			
L-Phenylalanine	150.00	1000.00	150.00
L-Proline	350.00	500.00	350.00
L-Hydroxy-L-Proline	–	800.00	–
DL-Serine	1100.00	400.00	–
L-Serine	–	–	550.00
L-Threonine	175.00	200.00	180.00
L-Tryptophan	100.00	100.00	100.00
L-Tyrosine	50.00	–	55.00
L-Tyrosine 2Na $2H_2O$	–	(360.40)[b]	72.08
L-Valine	100.00	500.00	100.00
Vitamins			
Para-aminobenzoic acid	0.02	0.32	0.02
Biotin	0.01	0.16	0.01
D-Ca pantothenate	0.02	0.008	0.11
Cholin chlorid	0.20	20.00	–
Folic acid	0.02	0.08	0.02
iso-Inositol	0.02	0.40	0.02
Niacin	0.02	–	–
Nicotinic acid	0.02	0.16	0.02
Pyridoxine HCl	0.02	0.40	0.02
Riboflavin	0.02	0.08	0.02
Thiamine HCl	0.02	0.08	0.02
Vitamin B12	–	0.24	0.01
Tryptose	–	–	2600.00
Lactalbumin	(3300.00)[c]	–	–
Yeast extract	(3300.00)[c]	–	–

[a] Compositions according to the Gibco Cell Culture Cataloque, 1996/1997.
[b] Powder medium.
[c] Hink's TNM-FH (1970).

lacking particular components such as amino acids, sugars and phosphates, the formulation should be made using x-fold stock solutions (Summers & Smith, 1987; O'Reilly *et al.*, 1992). All three media, which are generally supplemented with 10% FBS, are available in powder as well as in the more convenient liquid form, which is about five times more expensive. Generally the serum content can be reduced to 5% in these media without a significant decrease in cell growth and virus propagation.

Development of serum-free insect cell culture media

Insect cells, like Sf-cells, could be grown easily in the form of a stationary culture in T-flasks and in a suspension of a few liters, using conventional media supplemented with 8–10% FBS (Hink & Strauss, 1980, Miller *et al.*, 1986). However cell growth and the production of baculovirus and recombinant proteins on a large scale was difficult. Insect cells are reported to have a high demand for oxygen especially 14–20 h postinfection (Hink, 1982) and, unsually, they are highly sensitive to physical stress (Hink & Strauss, 1980, Tramper *et al.*, 1986). In order to scale up the insect cell culture for the production of baculovirus and recombinant proteins, these serious problems first had to be solved. Growing cells have to be protected against agitation and air bubbles in stirred and gas sparged bioreactors (Kilburn & Webb, 1968). Maiorella *et al.*, 1988, added Pluronic F–68, a cell protecting surfactant, to the insect culture medium. The protection of mammalian cells from air bubbles has been intensively investigated by Mizrahi (1975) and Handa *et al.*, 1987. In the absence of Pluronic F–68, cells were damaged during disengagement of bubbles at the liquid surface (formation of foam) in a sparged culture vessel. In the presence of the polyol, cells did not rise with the bubbles to the surface and the transfer of the gas from the liquid phase into the headspace did not form foam (Handa *et al.*, 1987). Addition of Pluronic F–68 effectively reduces the shear stress in sparged spinner and bioreactor cultures, and in addition functions as a potential antifoaming agent (Handa *et al.*, 1987; Murhammer & Gochee, 1988; Maiorella *et al.*, 1988). Pluronic F–68 is a block copolymer consisting of about 50% polyoxypropylene (hydrophobic) and 50% polyoxyethelene (hydrophilic). It is assumed that the hydrophobic part of the molecule interacts with cell membranes (Murhammer & Goochee, 1988). Pluronic F–68 is usually added at a concentration of 0.01% in vertebrate media (Velez *et al.*, 1986) and 0.1% in insect cell culture (Inlow *et al.*, 1989). The polyol does not interfere noticably with cell growth or purification procedures (Maiorella *et al.*, 1988).

Another difficulty in scaling up insect cell cultures is the serum supplemented medium. Serum serves as a source of growth promoting proteins (hormones) and of essential low molecular weight substances, mainly lipids, and other components which support mammalian cell growth (Iscove, 1984; Goodwin, 1991). Lipids are important components of cell culture media (King and Spector, 1981). Furthermore, serum factors protect cells from general stress induced by shear forces, air bubbles, and by toxic components which function in trace amounts, like metal ions and oxygen radicals (Handa et al., 1987; Goodwin, 1991).

However, the use of FBS has several serious disadvantages as well. FBS is very expensive; serum addition accounts for more than 50% of the total medium cost, and it is sometimes difficult to obtain. The quality of the serum can vary from lot to lot and its components can affect cell growth and virus replication. Serum proteins hinder the downstream processing of recombinant proteins in the culture supernatant (the purification procedure becomes less cost effective). FBS lots can be contaminated by adventitous agents and mycoplasma. These (old) arguments clearly show that serum is undesirable in large scale insect cell cultures.

Development of a serum-free medium has to include the substitution of essential growth supporting factors provided by fetal bovine serum (Iscove, 1984). Because of the well known difficulties which arise from the use of serum, various attempts have been made to develop serum-free media formulations which can support insect cell growth in suspension culture (Wu *et al.*, 1989). Many efforts have resulted in reduced growth and production properties (Mitsuhashi, 1989; Hink, 1991). Lipid factors have been used to replace serum in the culture of numerous cell lines (Spector *et al.*, 1981; Goodwin, 1989; Goodwin, 1991). Röder (1982) successfully cultured several Lepidoptera cell lines in the presence of 1 % egg yolk emulsion as a source of the required lipids (lecithins and cholesterol). Cell growth of Sf-cells and baculovirus replication in TC–10 supplemented with egg yolk was the same as in serum containing culture. Vaughn and Fan (1989) have tested two commercially available serum replacers, CPSR–2 and CPSR–3 (Sigma), which were successful in Sf21 cell culture with IPL–41 medium. However the growth promoting activities were not as good as in FBS.

In order to avoid other complex nutrient supplements based on protein, such as egg yolk, Maiorella and Inlow supplied lipids in the form of a microemulsion to the culture medium, to grow Sf9 cells on a fermentor scale (Maiorella *et al.*, 1988, Inlow *et al.*, 1989). Based on IPL–41, Inlow *et al.*, 1989, prepared the serum-free ISFM medium by adding ultrafiltered yeastolate ($4\ g\ l^{-1}$) and a complex lipid emulsion. The supply of lipids to culture media has proved to be problematic. Inlow *et al.*, 1989, overcame this serious proplem by dissolving cod liver oil fatty acid methyl esters and Tween 80, both low cost lipid sources,

cholesterol (sterol source), and α-tocopherol acetate (antioxidant) in ethanol. Latter substance was incorporate into nutrient liposomes to prevent membrane and liposomal lipid oxidation. Dropwise addition of 10% Pluronic F–68 (in water), while the lipid solution is vigorously mixed, results in a clear microemulsion (also see below the preparation of SF–1 medium). This lipid microemulsion was added to the culture medium before use. Besides the protection of cells against hydrodynamic stress, Pluronic F–68 serves as a detergent to dissolve the lipid-ethanol solution in a clear microemulsion (Maiorella *et al*., 1988). According to the detailed protocol described by Inlow *et al*., 1989, the preparation of the lipid emulsion is simple (see below).

Using ISFM medium, Sf9 cells (and other insect cell lines) could be grown in lab spinner flasks and in an airlift fermentor with resulting cell densities of 5–6 x 10e6 cells per ml. The utility of this sytem was demonstrated by producing high amounts of recombinant human macrophage colony stimulating factor (Maiorella *et al*., 1988). Inlow and Maiorella developed the ISFM medium and established the conditions for using the airlift fermentor system for insect cells which provided high oxygen supply at relatively low hydrodynamic shear forces, making production on a large scale possible. Other lipid emulsions are available from several suppliers (Inserum 1, JRH Biosciences, Lipid Concentrate, Gibco).

Meanwhile, other improved serum-free media mainly based on IPL–41 medium have become commercially available (O'Reilly *et al*., 1992). ExCell–400 is a semi-defined medium (containing 15 mg ml^{-1} protein or less) for the serum-free culture of lepidopteran insect cells (Belisle *et al*., 1992) and ExCell 401 medium is the improved protein-free version for high cell density growth and increased yields of expressed recombinant proteins (JRH, Biosciences). Another medium with a low protein content is Sf 900 (Weiss *et al*., 1992), and the improved second generation is the completely protein-free Sf 900 II medium (Gibco). ExCell 401 and Sf 900II are the most commonly used serum-free insect cell culture media at present. Detailed compositions of both media are not published by the supplier. An overview of some essential nutrients of the most common insect cell culture media which are available is given in Table 2. The comparison of the concentrations of glucose, glutamine and protein hydrolysates can be helpful. For example, from TC–100 medium it is evident that glucose is one of the limiting nutrients. Addition of 2–3 g l^{-1} is recommended. IPL–41 and Grace's medium contains additional hexoses. Furthermore the amount of protein lysate is probably too low. Addition of yeast extract (0.2–0.4%) would make TC–100 more nutrient-rich and useful not only for a static culture but for suspension culture as well.

The development of serum-free culture media has been of great value to the large scale production process. It should be noted reallistically, however, that in lab cultures, where only small quantities are needed, high serum addition will continue in many labs because the cultures are easier to handle. As an alternative, reduction of the serum content from 10% to 1.0 (0.5)% in the new media which are available for protein-free growth is highly recommended. The low serum concentration provides enough growth promoting activity, the overall cost is significantly reduced and the cells are protected to some extent against stress as well as recombinant proteins against proteolytic attack is ensured. In conclusion, based on the IPL–41 medium, a new set of serum-free insect cell culture media have been developed which support the growth of baculovirus in Lepidoptera derived cell lines and which allow the scaling-up process to be developed further.

Development of the low-cost SF–1 medium

Batch to batch variation, difficulties with delivery and a significant price increase in the first generation of commercially available serum-free insect cell culture media, prompted us to investigate the possibility of developing a cheap and reproducible insect cell medium which would be particularly useful for the production of recombinant proteins in a scaled-up fermentation (Schlaeger *et al*., 1992A). We ended up with the semi-defined IP301 medium which is a modification of IPL–41, and SF–1, which is a low-cost medium based on ultrafiltered protein hydrolysates (Schlaeger *et al*., 1993). In SF–1 medium, expensive amino acids and vitamins are replaced by three different protein hydrolysates. No amino acids other than glutamine or vitamins were added. Both serum-free media have to be supplemented with the lipid microemulsion mixture according to Inlow *et al*., 1989. The growth properties of both media for Sf9 and Sf21 cells are very similar (maximum cell density 8–11×10E6 cells ml^{-1}, PDT = 22–24 h; Schlaeger *et al*., 1993). However, SF–1 medium has several major advantages over IP301, for example simple and rapid preparation, very cost-effective, and therefore particulary useful for the large

Table 2. Essential supplements for commonly used insect cell culture media

Components		TNM-FH (Grace)	TC–100	IPL–41	Ex-Cell 401	Sf900 II	SF–1
		(serum-dependent)			(serum-free)		
Glucose	g l^{-1}	0.7	1	2.5	2.5	2.4	10.0	7.5
Glutamine	mM	4.1[a]	4.1[a]	6.85[a]	6.85[a]	5.5[a] (6.8)	10.0[a]	7.5[a]
Lactalbumin	%	0.33	–	–	–	?	?	0.56
Yeast extract	%	0.33	–	–	0.4	+	+	0.56
Tryptose	%	–	0.26	–	–	?	?	–
Primatone RL	%	–	–	–	–	–	–	0.56
FBS	%	5–10	5–10	5–10	–	0	0	0
Lipids		–	–	–	+[b]	+	+	+[b]
Pluronic F–68	%	–	–	–	0.1	+	+	0.1

[a] Our result from HPLC analysis, ( ) value from JRH Biosciences, Cat. Nov. 1992.
[b] Lipid microemulsion according to Inlow *et al.*, 1989.

scale production in batch or perfusion mode. Therefore SF–1 has been the insect cell culture medium of choice in our laboratory and fermentor cultures for years. In addition, the use of the nutrient rich SF–1 as plaque medium facilitate the determination of plaques.

After an adaptation phase of several passages, Sf9 cells grow in SF–1 medium to a cell density of 5–6 × 10E6 cells ml^{-1} with a PDT of 26–28 h. However, addition of 10% IP301 medium improved the cell growth and production properties significantly (Schlaeger *et al.*, 1993). This formulation has been used for years for the passage of different insect cell lines and for the production of recombinant proteins for research purposes on a lab as well as fermentor scale (231,601). It should be noted that the addition of 10% IP301 to improve SF-1 medium can be replaced by other insect cell culture media. Grace's, IPL–41, Ex-Cell 400 and Sf900 II medium were studied and all gave similar growth properties to those of IP301. To reduce the total cost of SF–1 medium for large scale production an amino acid-free and vitamin-free version of this additional medium is recommended. In spite of the same growth properties in the absense of serum, the addition of 0.5 (–1)% FBS was normally used to satisfy the product quality during infection because protein degradation correlates to the cell viability at the harvest point, which varies.

The combination of lactalbumin, yeast extract and Primatone RL provides an excellent composition of nutrients to support cell growth and baculovirus production. Primatone RL is an enzymatic digest of "US"-meat and has been used for a long time to reduce or even to replace fetal calf serum in vertebrate media for cultures of human and rodent cells, with no reduction in performance (Zoon *et al.*, 1979). Many years of experience with the low cost nutrient sources in our laboratory has shown that addition of Primatone RL to serum-free HL- or DHI-medium allows growth to high cell density and prolonges the viability of several human and rodent cells in suspension culture (Schumpp & Schlaeger, 1990; Schlaeger & Schumpp, 1992). More recently we have been able to show that mouse hybridoma cells grown in serum-free DHI medium supplemented with Primatone RL, which was also used to feed the culture during the so-called stationary phase, produce high amounts of antibodies in fed-batch cultures (Schlaeger & Christensen, 1996; Schlaeger, 1996). Therefore we used Primatone RL in SF–1 medium, together with lactalbumin and yeast extract, to enrich the basic nutrient sources for Sf9 cell growth, mixing it with a salt solution derived from conventional insect cell culture media.

Before protein hydrolysates were ordered, several lots of each lysate were tested for their growth promoting properties with Sf9 cells. A price comparision of different suppliers is recommended. Stock solutions of 10% were made by dissolving the hydrolysate powder (for 50 l) in water followed by ultrafiltration through a cassette membrane, cut-off 8 K, 1–2 h, and a sterile filtration through a 0.2 micron Durapore® filter. The sterilized hydrolysates can be stored for over 2–4 months at 4 °C. The ultrafiltration step removes possible contaminating proteins and other high molecular weight substances and is probably responsible for the reproducible growth supporting activities of these complex nutrient sources. Therefore, ultrafiltration of

protein hydrolysates from different sources is highly recommended for use in the cell culture. Yeast extract and lactalbumin powder can be dissolved together, if necessary at slighly higher concentrations (12.5–15%). Recently a white precipitate was regularly observed in several Primatone RL batches after solubilization in water. Introduction of a continuous centrifugation step (Heraeus Contifuge, 15000 rpm, 1–2 h, 50 1) followed by a prefiltration step (1 micron filter) before the ultrafiltration treatment solved this problem. Some information on the compositions of all three protein hydrolysates is listed in Table 3. About 50% of the amino acids content of average Primatone RL and lactalbumin batches and 75% of yeastolate occur in the form of oligopeptides. The free amino acid concentrations in the culture medium can vary during growth and infection, due to the cleavage activity of released proteases (Hansen & Emborg, 1992). This has to be taken into account if amino acid consumption rates or even final consumptions (expressed as percentage of the initial content in culture medium) are determined.

Recently a slight modification to the SF–1 medium was introduced to make it more useful for high cell densitiy infection, production of recombinant proteins and for the nutrient requirements of High Five cells. Furthermore, the Drosophila Schneider cell line SL–3 was adapted to grow to high cell density in this improved SF–1 medium (Schlaeger, unpublished). The Sf-cell lines (Sf9, Sf21), High Five and the Drosophila melanogaster SL–3 (SL–2) cells were easely adapted to serum-free growth in SF–1 medium within 5–15 passages. The preparation of fortified SF–1 medium, which is used presently in our laboratory and production cultures, is shown in Table 4. The concentration of glucose and glutamine was increased from $5\ g\ l^{-1}$ and 5 mM to $7.5\ g\ l^{-1}$ and 7.5 mM respectively. In addition, the amount of the protein hydrolysates was increased giving the final amino acids concentrations as shown in the right column of Table 4. No antibiotic is included in the SF–1 medium. The use of antibiotics is generally not recommended either in laboratories cultures or in large scale fermentation. Microbial contamination can be detected more quickly. No antifoam agent is used in the fermentation cultures since this may interfere with the subsequent purification procedure.

In order to complete the description of the preparation of SF–1 medium, the lipid microemulsion preparation of Inlow *et al.*, 1989, is described again in Table 5. Its successful mixing is highly dependent on temperature (37 °C), dropwise addition and high speed vortexing. If these points are taken into account initially, the preparation is simple. We used this lipid mixture routinely for all laboratory cultures and large scale productions because it is cost effective and it is at least as good, if not better than other commercially available lipid emulsions for our purposes.

Future aspects

The development of insect cell culture media has been relatively successful during the last three decades, especially for cell lines from the Lepidoptera type which are used increasingly in biological research (expression of recombinant proteins) and production of bioinsecticides (baculovirus). The handling of suspension cultures of Sf-cells has become easier and high cell densities of 10–14 × 10E6 cells ml^{-1} or even more (Radford *et al.*, 1995) in serum-free media have been achieved. These cell liters are generally two to three times higher than the average well growing mammalian cells, like mouse hybridoma cell lines. Furthermore, in a few cases large scale fermentor production was possible in serum-free culture medium at a cost effective price. What is coming next? Is the near future just a period of fine tuning of existing media or will there be another breakthrough in design of cell culture medium (using genetic engineered cell lines, for instance)? Three aspects will be discussed here. 1. Development of a general base medium for many different insect cell lines. 2. Specific cell growth factors and essential lipids. 3. Specific nutrient requirements during infection.

1. Presently, not all insect cell lines from different species can be grown well in cell culture using the existing media. Some cells may have very specific requirements. In the future, however, only a few formulations of cell culture media will be used for the growth of the majority of invertebrate cell lines, especially those cell lines which propagate baculovirus in vitro. From vertebrate cell culture it is known that under various altered physical and physiological conditions mammalian cells can respond and survive by changing their metabolism, if a certain adaptation period is allowed. For example, mouse hybridoma cells are able to grow under conditions of high agitation stress without being damaged if the cells have been adapted over a long culture period with increasing stirrer speed (Schmid *et al.*, 1992). In addition, isolation of lactate-ammonia double resistant human HL–60 cell clones were grown in culture medium contain-

Table 3. Physicochemical properties of hydrolyzed proteins, typical values[a]

Protein hydrolysates	Free amino acids	MW 100–200 Daltons	MW 200–500 Daltons	MW 500–1000 Daltons	MW average Daltons	Total amino acids
	(%)					($mg\ g^{-1}$)
Primatone RL	53	64.6	30.9	4.5	206	705.1
Lactalbumin[b]	54	21.7	42.2	27.7	384.5	817.4
Yeast extract	25	38	40	17	325	603.5

[a] Sheffield Series, Technical Manual, Quest International.
[b] Edamin.

Table 4. SF–1 medium (serum-free)

Preparation of 1L SF–1 Components	Concentrations		Free amino acids Concentrations (HPLC analysis values)	
	mg	ml^{-1}		$mg\ l^{-1}g$
H_2O (nanopur quality)		850	L-Alanine	437.0
IP301 powder[a]	1865		L-Arginine	438.0
Salt solution			L-Asparagine	529.0
KCl	2900		L-Aspartic acid	331.0
NaH_2PO_4 (anhydrous)	1000		L-Cystein	83.0
$MgCl_2$ (anhydrous)	1062		L-Glutamine acid	705.0
$MgSO_4$ (anhydrous)	1358		L-Glutamine	1096.0
$CaCl_2$	1000 (750)		Glycine	225.0
$NaHCO_3$	350		L-Histidine	236.0
L-Glutamine	1023		L-Isoleucine	392.0
L-Methionine	53.8		L-Leucine	883.0
L-Cystine. 2HCl	43		L-Lysine	810.0
L-Asparagine	264		L-Methionine	376.0
Cholin chloride	37.6		L-Phenylalanine	478.0
Inosine	26.9		L-Proline	4.2
Glucose	7000		Hydroxy-L-Proline	62.5
Lactalbumin (10%) (1)		56.25	L-Serine	326.0
Primatone RL (10%) (2)		56.25	L-Threonine	283.0
Yeast extract (10%) (3)		45.00	L-Tryptophan	162.0
			L-Tyrosine	149.0
			L-Valine	411.0

(Sf–900 II powder, 3865 mg (Gibco)).
Adjust pH to 6.2–6.4 (HCl), adjust osmolality to 340 mOsmol kg^{-1} (NaCl), filter sterile through a 0.2 micron filter (Durapore® membrane, Millipore).
(1) UF-filtered (8K cut-off), Gibco (or Difco), (2) UF-filtered (8K cut-off) Sheffield (Quest International), (3) UF-filtered (8K cut-off) Gibco (or Difco).
[a] Amimed (BioConcept), CH–4123 Allschwil 3, Switzerland.

ing a highly toxic concentration of both metabolites (Schumpp & Schlaeger, 1990). Using a continuous growth culture of hybridoma cells under glutamine limitation, glutamine independent variants (induction of glutamine synthetase) arose with time and outgrew the culture (Birch *et al.*, 1994). These few examples of growth adaption under altered conditions demonstrate that vertebrate cells, especially established cell lines, can respond positively to external environmental changes. There is good evidence that invertebrate cells also possess such adaptive mechanisms and it should therefore be

Table 5. Preparation of the lipid microemulsion according to Inlow *et al.*, 1989

A.	Lipid solution in ethanol (stock solution for 100 l SF–1 medium)			
				(2-fold)
	Cholesterol	Sigma, C 2044	450 mg	(900 mg)
	α-Tocopherol acetate	Sigma, T 1157	200 mg	(400 mg)
	Cod liver oil	Sigma, C 2294	1000 mg	(2000 mg)
	Tween 80	Sigma, P 4675	2400 mg	(4800 mg)
	dissolve in 75ml ethanol (100%), warm to 37 °C, vortex, add 25 ml ethanol, ethanol, filter sterile through a 0.2 micron Vacucap filter (Gelman Sciences), store in small glass flasks at –20 °C, protect for light.			
B.	Pluronic F–68 solution (10 % in water). Serva, Cat.no. 35724, Synperonic F–68 pract. MW 83000 Mix slowly 100g powder in 800 ml cell culture grade water for 10 min at 20 °C, stir additional 60–90 min, adjust to 1 l volume with water, filter sterile through a 0.2 micron Vacucap filter (Gelman Sciences) and store at 4 °C.			
C.	Lipid microemulsion for 1 l medium. Warm lipid solution, Pluronic F–68 solution and medium in a 37 °C waterbath. Add under sterile condition 1 ml lipid solution (A) into a 50 ml screw cap tube and add dropwise (slowly, 1 min) 10 ml Pluronic solution (B) while the tube is vigorously mixed on a vortex. The final emulsion should be clear–to-opalescent. Add 11 ml lipid emulsion to 1 l prewarmed medium under shaking. For 10 l scale 100 ml Pluronic F–68 (B) are dropped slowly to 10 ml lipid solution in a 250 ml conical tube (Corning, 10 min). In case of the 2-fold lipid solution use 0.5ml lipids add only 5 ml Pluronic F–68 solution (B) slowly, the emulsion should also be clear-to-opalescent, further 5 ml Pluronic solution is added to obtain finally 0.1% concentration Pluronic in the medium.			

possible to exploit this flexibility. Thus it should be possible to adapt many insect cell lines from different species under suitable conditions to a few culture media.

2. Specific requirements have to be taken into account in isolating more cell lines from different insects in order to find more candidates for research and production purposes. In this respect the hemolymph of the different insects has to be used as a source of factors with growth and maintainance potential (Ferkovich & Oberlander, 1991). These factors have to be isolated and characterized for their use in cell culture. Little effort has been made so far in identifying such factors in invertebrates. The general difficulties should not necessarily be greater for insects than for vertebrates. Recently two iron binding proteins have been described, one high molecular weight protein (490 kDa) functionally comparable to mammalian ferretin and a 80 kDa protein similar to transferrin (Hubners *et al.*, 1988). Furthermore, molecular biological studies revealed nucleotide sequences which predict evidence of insect (Drosophila) epidermal growth factor (EGF) and a **TGF-β-like** protein, as well as insulin and steroid hormone receptors (Wharton *et al.*, 1985; Padgett *et al.*, 1987; Hursh *et al.*, 1987; Muskavitch & Hoffmann, 1990; Hide, 1990;). However, addition of vertebrate growth factors did not effect insect cell growth (Ferkovich & Oberlander, 1991). So far only a few invertebrate cell cultures have demonstrated any response to mammalian related hormone complexes.

As discussed before, the use of lipids in insect culture media is important in fulfilling the requirement for sterols and essential (and nonessential) polyunsaturated fatty acids in an oxidation-protected form (Goodwin, 1991). Although many existing mammalian and insect cell lines can be grown in lipid-

free culture media, most invertebrate and vertebrate species are not capable of synthesising essential fatty acids (Spector *et al.*, 1981; Goodwin, 1991). Therefore essential lipids are generally supplemented in insect culture media. However, it may be necessary to determine the essential lipid requirements for each cell line in detail, in order to optimise concentrations. Tompkins *et al.*, 1991 recently demonstrated that steroid-phopholipid supplementation could prevent the usual decrease in virulence of baculovirus serially propagated in cell culture. The importance of lipid metabolism in the viral-host system could be shown in several vetebrate studies which suggest that virus cell fusion, viral pathogenicity, and host range may be controlled by the host membrane lipid composition (Roos *et al.*, 1990; Guinea & Carrasco, 1990). The field of essential lipid mixture supplementation to culture media should be carefully investigated in the near future.

3. Many recombinant proteins are well expressed in the baculovirus expression system using Sf-cells in suspension cultures. To design improved culture media for this system, several culture parameters must be assessed. (1) Cell growth characterized by : population doubling time (PDT), growth rate (μ), final cell density (hcd), and perhaps also the length of the so-called stationary phase (h). (2.) Cell infection characterized by: volumetric extracellular virus titer (NOV's; pfu ml^{-1}), the specific virus liter (pfu/cell), amount of occlusion bodies (OV's), postinfection time-viability (h), and the volumetric yield of released recombinant proteins. Unfortunately, there is link between all these parameters. An increase of growth promotion generally correlates with enhanced productivity. However, improvements which lead to an increase in cell density from about 8 × 10E6 cells ml^{-1} to 12–14 × 10E6 cells ml^{-1} do not correlate well with an increased yield of recombinant proteins and extracellular baculoviruses (pfu ml^{-1}) after infection at a cell tiler of 2–3 × 10E6 cells ml^{-1} (Schlaeger, unpublished). Therefore, it can be assumed that a culture medium optimized for growth to high cell density does not necessarily fulfil all the requirements for an optimal production process. Another problem has to be addressed, namely that with increasing cell numbers the expression level of many proteins (if not all) in a batch culture decreases (Schlaeger *et al.*, 1992A). The reason for this is not well understood. Therefore only low cell numbers (1–3 × 10E6 cells ml^{-1}) are commonly used for protein production (Maiorella *et al.*, 1988; Schlaeger *et al.*, 1993). Efforts have to be made to achieve a belter understanding of the metabolic requirements of infected insect cells during postinfection. Additional component, which affect the production process in a positive way could be added to the growth medium. On the other hand, it appears more reasonable to create a special feed mixture containing additional base nutrients like glucose, amino acids, protein hydrolysates, lipids etc., and specific components to support virus propagation and protein expression, at least in high cell density infection cultures. This mixture has to be fed (once a day) during infection.

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References

Amborski RL & Moskowitz M (1968) The effects of low molecular weight materials derived from animal tissues on the growth of animal cells in vitro. Exptl. Cell Res. 53: 117–128.

Bédard C, Tom R, Kamen A & André G (1992) Nutrient consumption and waste product accumulation in Sf9 insect cell culture. In: Baculovirus and Recombinant Protein Production Processes (pp. 339–360). JM Vlak, E-J Schlaeger & AR Bernard (eds). Editiones Roche, Basel, Switzerland.

Belisle BW, Celeri C, Tang K, Montgomery T & Gong T (1992) From shake flask to large scale: Cell and virus production in serum-free media. In: Baculovirus and Recombinant Protein Production Processes (pp. 226–233). In: Vlak JM, Schlaeger E-J & AR Bernard (eds). Editiones Roche, Basel, Switzerland.

Birch JR, Boraston RC, Metcalfe H, Brown ME, Bebbington CR & Field RE (1994) Selecting and designing cell lines for improved physiological characteristics. Cytotechnology 15: 11–16.

Butler M & Jenkins H (1989) Nutritional aspects of the growth of animal cells in culture. J. Biotechnol. 12: 97–110.

Butler M (1992) Serum-free media for neuronal cell culture. In: Neuronal Cell Lines. A Practical Approach (pp. 55–75). JN Wood (ed.). Oxford University Press, Oxford.

Cameron R, Possee RD & Bishop DH (1989) Insect cell culture technology in baculovirus expression systems. TIBTECH 7: 66–70.

Davies AH (1994) Current methods for manipulating baculoviruses. Bio/Technology 12: 47–50.

Davis TR, Wickham TJ, McKenna KA, Granados RR, Shuler ML & Wood HA (1993) Comparative recombinant protein production of eight insect cell lines. In Vitro Cell Dev. Biol. 29A: 388–390.

Ferkovich SM & Oberlander H (1991) Growth factors in invertebrate in vitro culture. In Vitro Cell. Biol. 27A: 483–486.

Gardiner GR & Stockdale H (1975) Two tissue culture media for production of Lepidopteran cells and nuclear polyhydrosis viruses. J. Invertebr. Path. 25: 363–370.

Ginsberg HS, Gold E & Jordan WS (1955) Tryptose phosphate broth as supplementary factor maintenance of He La cell tissue culture. Proc. Soc. Exp. Biol. Med. 89: 66–71.

Glacken MW, Fleischaker RJ & Sinskey AJ (1986) Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnol. Bioeng. 28: 1376–1389.

Goodwin RH (1975) Insect cell culture: Improved media and methods for initiating attached cell lines from the Lepidoptera. In Vitro 11:396.

Goodwin RH & Adams JR (1980) Nutrient factors influencing viral replication in serum-free insect cell line culture. In: Invertebrate Systems in Vitro (pp. 493–509). Kurstak E, Maramorosch K & Dubendorfer A (eds). Elsevier/North Holland Biomedical Press, Amsterdam.

Goodwin RH (1985) Growth of insect cells in serum-free media. In: Techniques in the Life Science, Cell Biololgy. Vol C1, Techniques in setting up and maintenance of tissue and cell cultures (C109, pp. 28). Elsevier Scientific Publishers Ireland Ltd.

Goodwin RH (1989) Construction of peptoliposomes for the incorporation of nutrient lipid supplements in insect cell culture media. J. Tissue Culture Meth. 12: 17–20.

Goodwin RH (1991) Replacement of vertebrate serum with lipids and other factors in the culture of invertebrate cells, tissues, parasites, and pathogens. In Vitro Cell. Dev. Biol. 27A: 470–478.

Grace TDC (1962) Establishment of four strains of cells from insect tissue grown in vitro. Nature 195: 788–789.

Grace TDC (1982) Development of insect cell culture. In: Invertebrate Cell Culture Applications (pp. 1–8). K Maramorosch (ed.), Academic Press. New York.

Guinea R, & Carrasco 1 (1990) Phospholipid biosynthesis and poliovirus genome replication, two coupled phenomena. EMBO J.9:2011–2016.

Handa A, Emery AN & Spiers RE (1987) On the evaluation of gas-liquid interfacial effects on hybridoma viability in bubble column bioreactors. Delvelop. Biol. Standard 66: 241–252.

Hansen HA & Emborg C (1992) Complex medium supplements give difficulties when investigating mammalian cell physiology. In: Animal Cell Technology : Developments, Processes & Products (pp. 248-250). Spier RE, Griffiths JB & MacDonald C (eds), Butterworth-Heinemann Ltd, Oxford.

Hassell T & Butler M (1990) Adaption to non-ammoniagenic medium and selective substrate feeding lead to enhanced yields in animal cell cultures. J. Cell Sci. 96: 501–508.

Hide G (1990) Identification of an EGF receptor homologue in trypanosomes. In: Parasites, molecular biology, drug and vaccine design; UCLA symposia on molecular and cellular biology, new series, Vol. 130 (pp. 213–224). Agabian N & Cerami A (eds). Wiley–Liss, New York.

Hink WF (1970) Established cell line from the cabbage looper, Trichoplusia ni. Nature 226: 466–467.

Hink WF & Strauss EM (1980) In: Invertebrate tissue culture (pp. 297–300). Kurstak E & Maramorosch K (eds). Academic Press, New York.

Hink WF (1982) Production of Autographa californica nuclear polyhydrosis virus in cells from large- scale suspension cultures. In: Microbial and Viral Pesticides (pp. 493–506). Kurstak E (ed). Marcel Dekker, New York.

Hink WF (1991) A serum-free medium for the culture of insect cells and production of recombinant proteins. In Vitro Cell. Dev. Biol. 27a: 397–401.

Hsueh HW & Moskowitz M (1972) A growth factor for animal cells derived from peptone. Exptl. Cell Res. 77: 376–382.

Hubners HA, Hubners E & Webb BA (1988) Iron binding proteins and their roles in the tabacco hornworm, Manduca sexta (L). J. Comp. Physiol. B158: 291–300.

Hursh DA, Andrews ME & Raff A (1987) A sea urchin gene encodes a polypeptide homologous to epidermal growth factor. Science 237: 1487–1490.

Inlow D, Shauger A & Maiorella B (1989) Insect cell culture and baculovirus propagation in protein-free medium. J. Tissue Culture Meth.12: 13–16.

Iscove NN (1984) Culture of lymphocytes and hemopoietic cells in serum-free medium. In: Cell Culture Methods for Molecular and Cell Biology, vol. 4 (Methods for serum-free culture of neuronal and lymphoid cells, pp. 169–185). Barnes DW, Sirbascu DA & Sato GH (eds). Alan R. Liss, Inc., New York.

Jones BM & Cunningham I (1961) Growth by cell division in insect tissue culture. Exp. Cell Res. 23: 386–401.

Kilburn DG & Webb FC (1968) The cultivation of animal cells at controlled dissolved oxygen partial pressure. Biotechnol. Bioeng. 10: 801-814.

King ME & Spector AA (1981) Lipid metabolism in cultured cells. In: The growth requirements of vertebrate cells in vitro (pp. 293–312). Waymouth C, Ham RG & Chappie PJ (eds). Cambridge University Press, Cambridge.

Law JH & Wells MA (1989) Insects as biochemical models. J. Biol. Chem. 264: 16335–16338.

Ljunggren J & Häggström 1 (1992) Glutamine limited fed-batch culture reduces the overflow metabolism of amino acids in myeloma cells. Cytotechnology 8: 45–56.

Luckow VA & Summers MD (1988) Trends in the development of baculovirus expression vectors. Bio/Technology 6: 47–55.

Luckow VA (1991) Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: Recombinant DNA Technology and Applications (pp. 97–152). Prokop A, Bajpai RK & Ho CS (eds). McGraw-Hill Inc, New York.

Luckow VA (1993) Baculovirus systems for the expression of human gene products. Curr. Opinion Biotechnol. 4: 564–572.

Maiorella B, Inlow D, Shauger A & Harano D (1988) Large-scale insect cell-culture for recombinant protein production. Bio/Technology 6: 1406–1410.

Miller DW, Safer P & Miller LK (1986) An insect baculovirus host-vector system for high-level expression of foreign genes. In: Genetic Engineering, Vol. 8, Principles and Methods (pp. 277–298). Setlow JK & Hollander A (eds). Plenum Publishing Corp., New York.

Miltenburger HG & Krieg A (1984) Bioinsecticides: II. Baculoviridae. In: Advances in Biotechnological Processes 3 (pp. 291–313). Alan R. Liss, Inc., New York.

Mitsuhashi J & Maramorosch K (1964). Leafhopper Tissue Culture : Embryonic, Nymphal and Imaginal Tissues from Aseptic Insects. Contrib. Boyce Thomson Inst. 22: 435–460.

Mitsuhashi J (1982) Media for insect cell cultures. In: Advances in Cell Cultures, vol 2 (pp. 133–197). Maramorosch K (ed.). Academic Press, New York.

Mitsuhashi J (1989) Simplified medium (MTCM–1601) for insect cell lines. J. Tissue Culture Meth. 12: 21–22.

Mizrahi A (1975) Pluronic polyols in human lymphocyte cell line cultures. J. Clin. Microbiol. 2: 11–13.

Murhammer DW & Goochee CF (1988) Scaleup of insect cell cultures: protective effects of Pluronic F–68. Bio/Technology 6: 1411–1418.

Muskavitch MAT & Hoffmann FM (1990) Homologs of vertebrate growth factors in Drosophila melanogaster and other invertebrates. In: Growth factors and development (Current topics in developmental biology, vol. 24). Nilsen-Hamilton M (ed.), Academic Press, San Diego, CA.

O'Reilly DR, Miller LK & Luckow VA (1992) Insect cell culture media. In: Baculovirus expression vectors. A Laboratory Manual (pp. 110–117).WH Freeman and Company. New York.

Padgett RW, St Johnston RD & Gelbart WM (1987) A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. Nature 325: 81–84.

Radford KM, Cavegn C, Bertrand M & Bernard AR (1995) The indirect effect of multiplicity of infection in baculovirus infected insect cell cultures on recombinant protein expression. Baculovirus and Insect Cell Gene Expression Conference. Pinehurst, N.C. (USA), March 26–30.

Röder A (1982) Development of a serum-free medium for cultivation of insect cells. Naturwissenschaften 69: 92–93.

Roos DS, Duchala CS & Stephensen (1990) Control of virus-induced cell fusion by host cell lipid composition. Virology 175: 345–357.

Schlaeger E-J & Schumpp B (1992) Propagation of mouse myeloma cell line J558L producing human CD4 immunoglobulin G1. J. Immunol. Meth. 146: 111–120.

Schlaeger E-J, Loetscher H & Gentz R (1992A) Production of recombinant soluble human TNF receptors. In: Baculovirus and Recombinant Protein Production Processes (pp. 201–208). Vlak JM, Schlaeger E-J & Bernard AR (eds). Editiones Roche, Basel, Switzerland.

Schlaeger E-J, Loetscher H & Gentz R (1992B) Production of recombinant soluble human TNF receptor using the baculovirus insect cell expression system. In: Animal Cell Technology : Developments, Processes & Products (pp. 562–568). Spier RE, Griffiths JB & MacDonald C (eds). Butterworth-Heinemann Ltd, Oxford.

Schlaeger E-J, Foggetta M, Vonach JM & Christensen K (1993) SF–1, a low cost culture medium for the production of recombinant proteins in baculovirus infected insect cells. Biotechnol. Techn. 7: 183–188.

Schlaeger E-J, Stricker J, Wippler J & Foggetta M (1996) Investigations of high cell density baculovirus infection using Sf9 and High Five insect cell lines in the low-cost SF-1 medium. In: Animal Cell Technology 'Developments towards the 21st Centur' (pp. 313–315). Beuvery EC, Griffiths JB & Zeijlemaker WP (eds). Kluwer Academic Publishers. Dordrecht/Boston/ London

Schlaeger E-J & Christensen K (1996) Improvement of mammalian cell fed-batch culture. In: Animal Cell Technology 'Developments towards the 21st Century' (pp. 855–857). Beuvery EC, Griffiths JB & Zeijlemaker WP (eds). Kluwer Academic Publishers Dordrecht/Boston/ London.

Schlaeger E-J (1996) The protein hydrolysate, Primatone RL, is a cost-effective multiple growth promoter of mammalian cell culture in serum-containing and serum-free media and displays anti-apoptosis properties. J Immunol. Meth., in press.

Schmid G, Huber F & Kerschbaumer R (1992) Adaption of hybridoma cells to hydrodynamic stress under continuous culture conditions. In: Animal Cell Technology : Developments, Processes & Products (pp. 203–205). Spier RE, Griffiths RB & MacDonald C (eds). Butterworth-Heinemann Ltd., Oxford.

Schumpp B & Schlaeger E-J (1990) Optimization of culture conditions for high cell density proliferation of HL–60 human promyelocytic leukemia cells. J. Cell Science 97: 639–647.

Smith GE, Summers MD & Fraser MJ (1983) Production of human beta interferon in insect cells infected with a baculovirus vector. Mol. Cell. Biol. 3: 2156–2165.

Spector AA, Mathur SN, Kaduce TL & Hyman BT (1981) Lipid nutrition and metabolism of cultured mammalian cells. Prog.Lipid Res. 19: 155–186.

Summers MD & Smith GE (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Bulletin No. 1555. Texas Agricultural Experimental Station,.

Taylor WG, Dworkin RA, Pumper RW & Evans VJ (1972) Biological efficacy of several commercially available peptones for mammalian cells in culture. Exptl. Cell Res. 74: 275–278.

Tomkins GJ, Dougherty EM & Goodwin RH (1991) Maintenance of infectivity and virulence of nuclear polyhydrosis viruses during serial passage in noctuid (Lepidoptera: Noctudae) cell lines. J. Econ. Entomol. 84: 445–449.

Tramper J, Williams JB, Joustra D & Vlak JM (1986) Shear sensitivity of insect cells in suspension. Enzyme Microb. Technol. 8: 33–36.

Vaughn JL (1968) A review of the use of insect tissue culture for the study of insect-associated viruses. Curr. Top. Microbiol. Immunol. 42: 108–128.

Vaughn JL, Goodwin RH, Tomkins GJ & McCawley P(1977) The establishment of two cell lines from the insect Spodoptera frugiperda (Lepipdoptera; Noctuidae). In Vitro 13: 213–217.

Vaughn JL & Fan F (1989) The use of commercial serum replacements for the culture of insect cells. In Vitro Cellular & Developmental Biology 25: 143–145.

Velez D, Reuveny S, Miller 1 & Macmillan JD (1986) Kinetics of monoclonal antibody production in low serum growth medium. J. Immunol. Methods 86: 45–52.

Vlak JM (1992) The biology of baculoviruses in vivo and in cultered insect cells. In: Baculovirus and Recombinant Protein Production Processes (pp. 2–10). Vlak JM, Schlaeger E-J & Bernard AR (eds). Editiones Roche, Basel, Switzerland.

Wharton KA, Johansen KM & Xu T (1985) Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell 43:567–581.

Weiss SA, Smith GC, Kalter SS & Vaughn JL (1981) Improved method for the production of insect cell cultures in large volume. In Vitro 17: 495–502.

Weiss SA & Vaughn JL (1986) Cell culture methods for large-scale propagation of baculoviruses. In: The Biology of Baculoviruses, vol.2 (pp. 63–67) Granados RR & Federici BA (eds). CRC Press, Inc., Boca Raton, Fla.

Weiss SA, Whitford WG, Godwin GP & Reid S (1992) Media design: Optimizing of recombinant proteins in serum-free culture. In: Baculovirus and Recombinant Protein Production Processes (pp. 306–314). Vlak JM, Schlaeger E-J & Bernard AR (eds). Editiones Roche, Basel, Switzerland.

Wood HA & Granados RR (1991) Genetically engineneered baculoviruses as agents for pest control. Annu. Rev. Microbiol. 45: 69–87.

Wu J, King G, Daugulis AJ, Faulkner P, Bone DH & Goosen MFA (1989) Engineering aspects of insect cell suspension culture: a review. Appl. Microbiol. Biotechnol. 32: 249–255.

Wyatt GR (1961) The biochemistry of insect hemolymph. Ann.u Rev. Entomol. 6: 75.

Wyatt SS (1956) Culture in vitro of tissue from the silkworm Bombyx mori. J. Gen. Physiol. 39: 841–852.

Yamane I & Murakami O (1973) 6,8-dihydroxypurine: A novel growth factor for mammalian cells in vitro, isolated from a commercial peptone. J. Cell Physiol. 81: 281–284.

Yunker CE, Cory J & Meibos H (1981) Continuous cell lines from embryonic tissues of ticks. In Vitro 17: 139–142.
Zoon KC, Bridgen PJ & Smith ME (1979) Production of human lymphoblastoid interferon by Namalwa cells cultured in serum-free media. J. Gen. Virology 44: 227–229.

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PART II

VIRUSES

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Baculovirus–insect cell interactions

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Introduction

The Baculoviridae are a family of large, enveloped DNA viruses that are characterized by rod-shaped nucleocapsids and relatively large double stranded DNA genomes. Baculoviruses are infectious only to arthropods, with the vast majority of permissive species falling within one Order (Lepidoptera) of the Class Insecta. Genomes of different baculoviruses range from approximately 80–180 kbp. The Baculoviridae contain two Genera, the Nucleopolyhedroviruses (or NPVs) and Granuloviruses (or GVs) (Volkman *et al*., 1995). Because it has been difficult to develop convenient cell culture systems for the propagation of GVs (Winstanley & Crook, 1993), most molecular, biochemical, and genetic studies have focused on the NPVs. Baculoviruses interact at many levels with the host cell; yet much remains to be discovered about these interactions. Few studies have examined the participation of host proteins in baculovirus infection processes. However, information on viral proteins, and their structures and roles in infection is accruing rapidly. The sequence of the genome of the *Autographa californica* Multicapsid Nuclear Polyhedrosis Virus (AcMNPV) was recently reported along with an extensive analysis of known and predicted baculovirus genes (Ayres *et al*., 1994). The AcMNPV genome contains 154 open reading frames (ORFs) encoding potential proteins of ≥ 50 amino acids. A map of the approximately 134 kbp AcMNPV genome and some genes which have been identified are shown in Figure 1. In addition to reviews included in the present volume, previous reviews have also described aspects of baculovirus structure and molecular biology (Blissard & Rohrmann, 1990; Friesen & Miller, 1986; Granados & Federici, 1986; King & Possee, 1992; Miller, 1995; O'Reilly *et al*., 1992b; Rohrmann, 1992). This review will examine selected topics with an emphasis on known and likely viral interactions at the cell and molecular level.

Baculovirus virions

A hallmark of the Baculoviridae is the production of two virion phenotypes, termed budded virions (BV) and occlusion derived virions (ODV) (Figure 2). These two virion phenotypes are produced at different locations in the cell, and at different times in the infection cycle. They serve distinctly different functional roles. In addition, BV and ODV enter cells by different mechanisms. BV are produced in the late phase of the infection cycle, when nucleocapsids bud from the surface of infected cells. Thus, the BV envelope is derived from the modified plasma membrane of the host cell. Very late in infection, nucleocapsids become enveloped within the nucleus to form the ODV. ODV are subsequently occluded within an occlusion matrix protein ("Polyhedrin" in the NPVs; "Granulin" in the GVs). Since nucleocapsids of both BV and ODV are produced in the nucleus, the nucleocapsids and viral DNA of the BV and ODV appear to be identical (Figure 2). Thus, BV and ODV differ primarily in composition of the envelopes and associated structures, and these differences result in different functional roles of the BV and ODV.

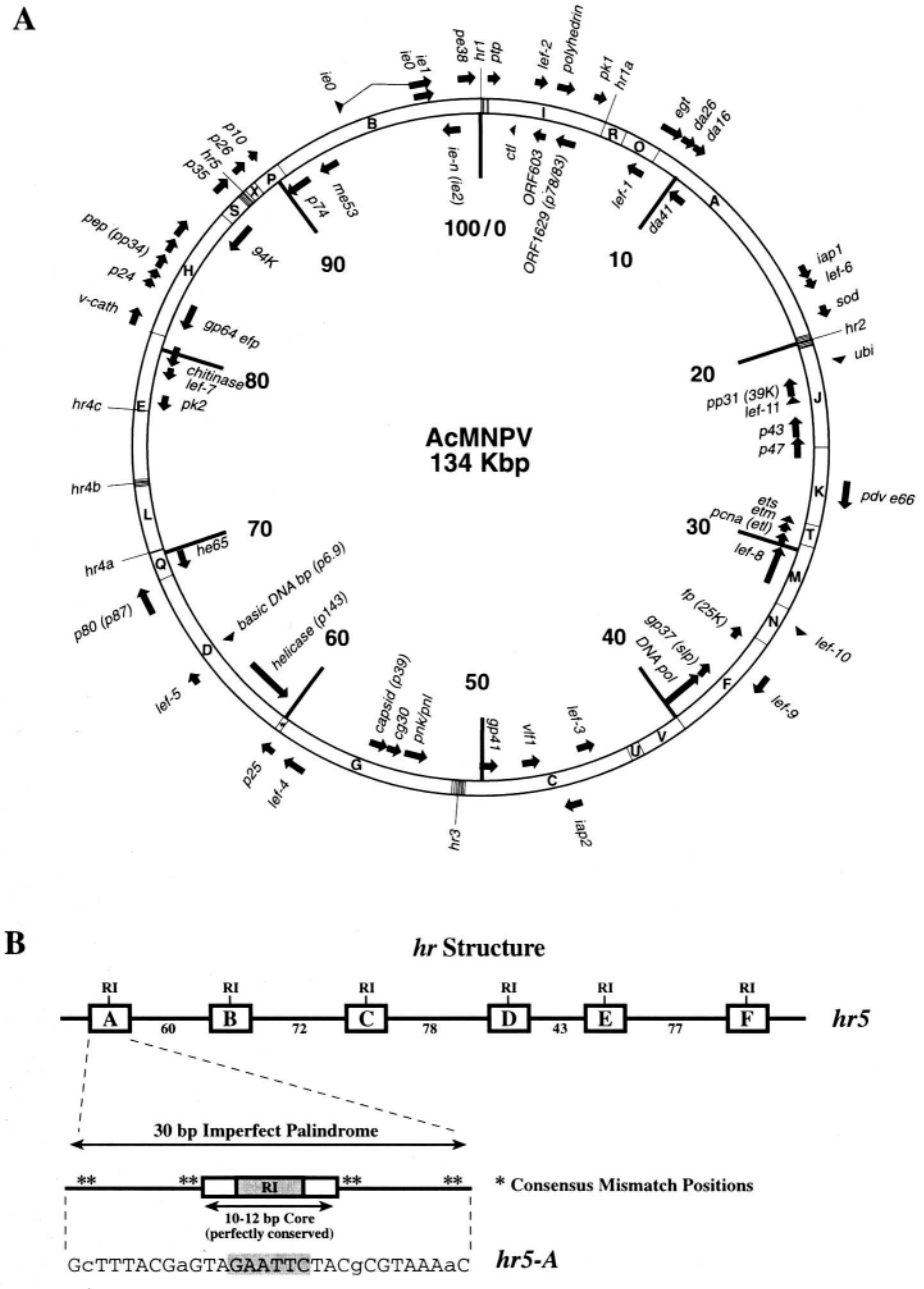

Figure 1. Organization of the AcMNPV genome. A. The approximately 134 Kbp circular dsDNA genome of AcMNPV is represented as a circle, with EcoRI sites and EcoRI fragments (A-X) indicated on the circle. The locations, orientations, and names of some of the known genes are indicated (thick arrows) along the inside and outside of the circle. Map units (0–100) are indicated by thick lines on the inside of the circle and the positions of homologous repeat (*hr*) regions are indicated along the outside of the circle. Map positions are from Ayres *et al.* (Ayres *et al.*, 1994). B. The structure of *hr* repeats within the AcMNPV genome are illustrated by a diagram of the *hr5* region. The top line represents the *hr5* region and the positions of the repeated 30 bp imperfect palindrome sequences are indicated as boxes (A-F). A single EcoRI site (RI) is located at the center of each 30 bp imperfect palindrome. The number of nucleotides between successive 30 bp imperfect palindrome repeats is indicated between the boxes. The structure of each 30 bp imperfect palindrome repeat is represented in the diagram below. The larger box represents the 10–12 bp perfectly conserved core sequence that is identical between all *hr* sequences and the inset gray box denotes the position of the EcoRI site (RI). Consensus mismatch positions within the palindrome are indicated (*). The sequence of the first 30 bp imperfect palindrome (*hr5-A*) within *hr5* is indicated below and the gray box indicates the EcoRI site at the center of the imperfect palindrome. Nucleotide mismatches in the *hr5-A* palindrome are in lowercase.

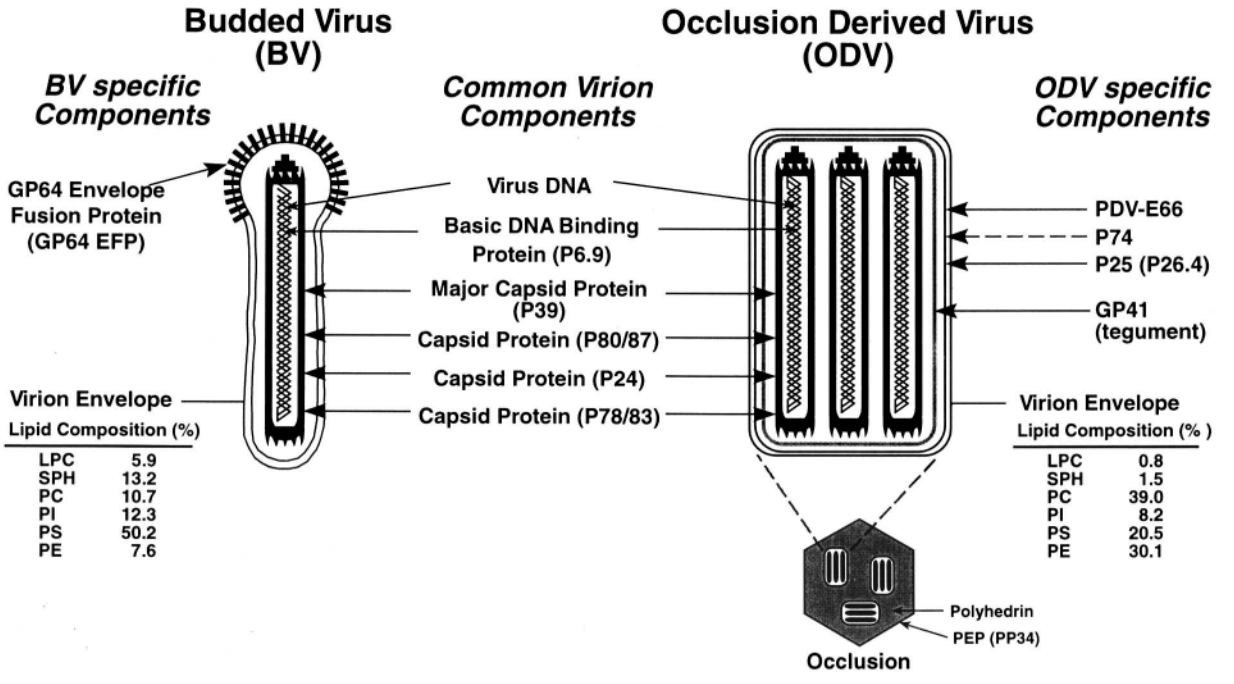

Figure 2. Structural composition of the two baculovirus virion phenotypes. The structures of the budded virus (BV) and occlusion derived virus (ODV) phenotypes are illustrated. The ODV structure in this illustration represents the multicapsid NPV group (see text). Proteins specific to either BV or ODV are indicated to the left and right of the diagrams. Proteins common to both virion phenotypes are indicated in the center. The polar nature of the baculovirus capsid is indicated in the diagram with the claw-like structure at the bottom and the ring-like nipple structure at the top of the capsid. Although the specific locations of most capsid proteins are not known, the P78/83 Capsid Protein was reported to be localized at one end of capsid (Vialard & Richardson, 1993). The possible location of the P74 protein is indicated by a dashed line. Lipid compositions of the BV and ODV envelopes derived from AcMNPV infected Sf9 cells (Braunagel & Summers, 1994) are indicated (LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine).

Biology and structure of ODV

In nature, baculovirus occlusion bodies are released into the environment after cell lysis and death of an infected insect host. New hosts acquire baculoviruses by feeding on foliage contaminated with viral occlusion bodies. After ingestion, occlusion bodies are exposed to the alkaline pH of the lepidopteran midgut, and this results in the dissolution of occlusion bodies and release of the occlusion derived virions (ODV). The ODV are highly infectious to epithelial cells of the insect midgut and appear to be less infectious in other tissues (Volkman & Summers, 1977). The number of nucleocapsids within an ODV envelope varies between different subgroups of baculoviruses. ODV may contain one or many nucleocapsids per envelope. GVs typically contain a single nucleocapsid per envelope and a single (ODV) virion per occlusion body (or capsule). In contrast, each NPV occlusion body contains many (ODV) virions. Historically, two subgroups have been described within the NPVs: Those typically containing a single nucleocapsid per virion are referred to as "single-capsid NPVs" (SNPVs), whereas those containing multiple nucleocapsids per virion are known as "multicapsid NPVs" (MNPVs). It is not clear whether the phenotypic differences observed between SNPVs and MNPVs represent significant phylogenetic differences between these groups since preliminary phylogenetic analyses of NPVs suggest that SNPVs and MNPVs do not segregate into separate groupings (Rohrmann, 1986; Zanotto *et al.*, 1993).

Do ODV contain viral encoded envelope proteins that interact with cellular receptors on insect midgut epithelial cells? Little direct data exist to address this question, and no specific virus-host cell receptor interaction has been identified for ODV. Electron micrographs of ODV do not reveal distinct spike, or peplomer structures on the surface of the envelope, as can be observed on some other enveloped viruses and on the BV of baculoviruses (see below). A recent study

of ODV (Horton & Burand, 1993), suggests that specific saturable virion binding sites exist on the brush border of midgut epithelial cells. However, specific interactions between viral encoded proteins and specific cellular proteins or other ligands have not yet been demonstrated.

After successful binding of the virion at the cell surface, nucleocapsids must enter the cytoplasm. Enveloped viruses normally enter cells by either direct membrane fusion at the cell surface, or by receptor mediated endocytosis. ODV enter cells by fusion of the virion envelope with the plasma membrane at the cell surface (Figure 3). Evidence for this mechanism of entry is largely from electron micrographic observations of midgut epithelial cells (Granados, 1978; Summers, 1971), and the finding that ODV entry is not inhibited by treatment with chloroquine, an agent that buffers the pH of the endosome and thus inhibits the entry of viruses by endocytosis (Horton & Burand, 1993). In the GVs, ODV infectivity is aided by a high molecular weight protein named "Enhancin" which is found in occlusion bodies (granules) (Gijzen *et al.*, 1995; Hashimoto *et al.*, 1991). The Enhancin protein has structural and functional characteristics of metalloproteases (R. Granados, pers. comm.) and the primary mode of action of Enhancin appears to be proteolysis of the peritrophic membrane, a structure that lines the insect midgut (Derksen & Granados, 1988; Wang *et al.*, 1994).

Information on the structural composition of the ODV is rapidly emerging. The ODV envelope, perhaps the most important component in the initial interaction of ODV with the host cell, contains a number of structural proteins. Viral encoded ODV envelope proteins include P25, PDV-E66, ODV-E56 (ODVP-6E) and possibly P74 (Braunagel & Summers, 1994; Braunagel et al., 1996; Hong *et al.*, 1994; Kuzio *et al.*, 1989; Roberts, 1989; Russell & Rohrmann, 1993a; Theilmann *et al.*, 1996) (Figure 2). An additional protein associated with ODV virions, GP41 (Liu & Maruniak, 1995; Ma *et al.*, 1993; Whitford & Faulkner, 1992a; Whitford & Faulkner, 1992b), is believed to localize in the "tegument" region. The tegument is a distinct region between the nucleocapsid and ODV envelope that has been observed in electron micrographs (Kawamoto *et al.*, 1977) (see Figure 2, tegument). The functional roles of ODV-specific structural proteins are largely unknown. However, occlusion bodies from an AcMNPV virus containing an inactivated *p74* gene are not infectious, suggesting that P74 plays an important role in ODV infectivity (Kuzio *et al.*, 1989). Neutralizing antibodies directed against specific ODV structural proteins have not been reported. In addition to the protein composition of the ODV envelope, the lipid composition is also likely to be a critical factor in virion infectivity and function. The mechanism of ODV envelope assembly in the nucleus is not known. However, the ODV envelope appears to be a typical lipid bilayer membrane and it has been suggested that the ODV envelope may be derived from invaginations of the inner nuclear membrane, forming microvesicles within the infected cell nucleus (Fraser, 1986b; Hong *et al.*, 1994). One ODV envelope protein, PDV-E66, was shown to localize to nuclear microvesicles, suggesting that nuclear microvesicles are the likely precursors of the ODV envelope (Hong *et al.*, 1994). A recent comparison of membranes from *Spodoptera frugiperda* Sf9 cell nuclei and envelopes from ODV and BV (of the AcMNPV baculovirus) showed significant differences in membrane lipid profiles (Braunagel & Summers, 1994). The composition of phospholipids from ODV and Sf9 nuclei differed quantitatively, for all classes of phospholipids examined except phosphatidylethanolamine. While phosphatidylcholine and phosphatidylethanolamine were the predominant phospholipids in the ODV, phosphatidylserine was the major phospholipid in Sf9 nuclei. Thus, lipid profiles of Sf9 cell nuclei and ODV differ significantly indicating that the ODV envelope, if derived from the nuclear envelope, appears to contain significant modifications.

Biology and structure of BV

Budded virions observed in electron micrographs typically contain a single rodshaped nucleocapsid which is surrounded by an envelope that has been described as a "loosely fitting" lipid bilayer membrane. Prominent spike-like structures or peplomers are often observed in the envelope, at one end of the mature virion (Figure 2). In addition, similar structures have been observed concentrated in the cellular plasma membrane at sites where budding occurs (Volkman, 1986). The major envelope protein of the BV is the GP64 Envelope Fusion Protein (GP64 EFP) (Blissard & Rohrmann, 1989; Whitford *et al.*, 1989), and this protein is not found in ODV. Immunoelectron microscopic studies of budding and mature virions indicate that the peplomers are composed of GP64 EFP (Volkman, 1986; Volkman *et al.*, 1984). Recently, the baculovirus transcriptional activator, IE1, was identified in BV but not ODV virions of the *Orgyia pseudotsugata* MNPV (OpMNPV) (Theilmann & Stewart, 1993). However, the location

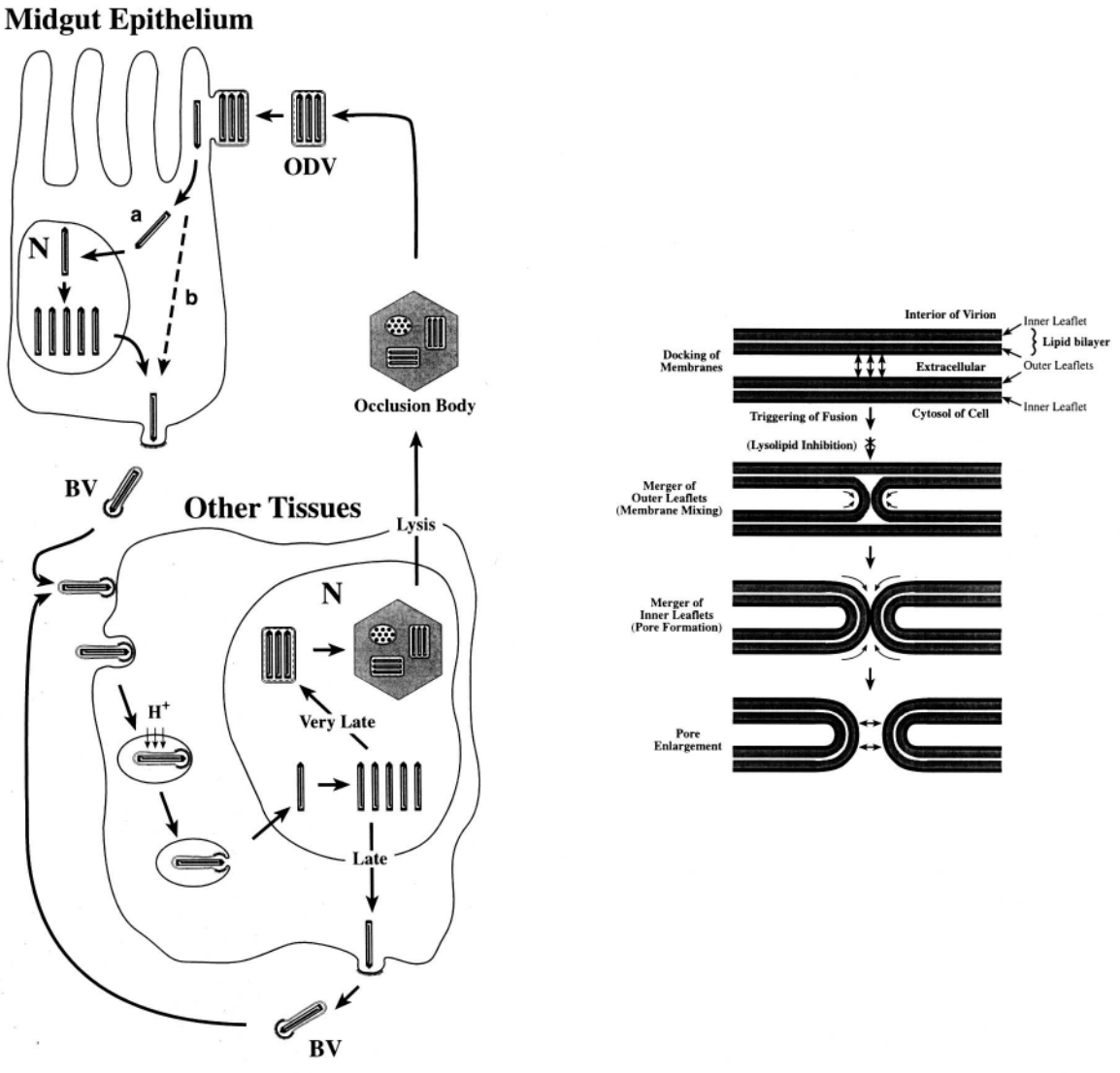

Figure 3. Baculovirus entry into host cells. A. Baculovirus infection cycle in midgut epithelial cells and other tissues of the insect. TOP PANEL. The top panel illustrates the infection of midgut epithelial cells by the occlusion derived virus (ODV) by fusion of ODV at the plasma membrane. Two possible routes through the midgut epithelial cells are indicated (a and b). In what is perhaps the typical route (a), nucleocapsids are transported to the nucleus where a round of replication occurs. Progeny virions subsequently bud from the basal side of the cell. Another possible mechanism (b) involves the immediate transport of some nucleocapsids to the basal side of the midgut epithelial cell and rapid budding. (Adapted from Granados & Lawler (1981).) BOTTOM PANEL. Budded virions enter other cells by endocytosis. After virion uptake into the endosome, the acidification (H^+) of the endosome triggers membrane fusion by the GP64 Envelope Fusion Protein (GP64 EFP). Nucleocapsids are then transported to the nucleus where gene expression, DNA replication, and assembly of progeny nucleocapsids occurs. During the late phase, nucleocapsids are transported to the plasma membrane where they bud to form BV. During the very late phase of infection, nucleocapsids are enveloped within the nucleus and are subsequently occluded within a crystalline matrix of the occlusion body protein, Polyhedrin. Occlusion bodies are released into the environment upon lysis of the cell. B. Model of membrane fusion and pore formation between viral and host membranes. Before nucleocapsids can enter host cells, virion and host membranes must fuse and form a pore (or pores) which subsequently widens to allow passage of the nucleocapsid. In the BV, membrane fusion is mediated by the GP64 EFP. In the ODV, a viral protein mediating membrane fusion has not been identified although the recent identification of ODV envelope proteins suggest several possible candidate ODV fusion proteins. A hypothetical model of steps involved in fusion of the two adjacent membranes is presented. Major steps are indicated on the left. The top panel shows two closely opposed lipid bilayer membranes that must first be "docked" or held in close apposition. In the BV, GP64 EFP mediated membrane fusion is triggered by exposure to low pH. Subsequent steps include the merger of outer membrane leaflets (membrane mixing), merger of inner leaflets (pore formation), and pore enlargement. Small arrows indicate the steps that are presumably catalyzed by viral membrane fusion proteins. The step at which lysolipids can inhibit membrane fusion is also indicated.

of IE1 in the virion is not known and it is currently unclear whether IE1 is present in the BV of all baculoviruses. A modified form of ubiquitin was also recently identified on the inner surface of the BV envelope (Guarino *et al*., 1995). This modified ubiquitin contains an unusual phospholipid anchor that likely results in the membrane association. Because modified ubiquitin in the BV is composed of both viral and host-encoded ubiquitins (Guarino, 1990; Guarino *et al*., 1995), further study will be necessary to determine whether ubiquitin plays a role in virion production or infectivity. The lipid composition of the BV envelope differs significantly from that of the ODV envelope (Figure 2) (Braunagel & Summers, 1994). In BV envelopes from AcMNPV propagated in Sf9 cells, phosphatidylserine is the major phospholipid, comprising approximately 50% of the total phospholipid content. In contrast phosphatidylcholine and phosphatidylethanolamine are the predominant phospholipids of the ODV envelope. Because lipid composition can dramatically influence membrane fluidity and perhaps the mobility of proteins, lipid composition is likely to be an important factor in the function of the two baculovirus virion phenotypes. Analysis of lipid compositions of the respective envelopes suggests that the BV envelope is more fluid than that of the ODV (Braunagel & Summers, 1994). In addition, the ODV envelope appears to contain higher concentrations of protein than the BV envelope.

Following ODV infection of midgut epithelial cells, infection is extended to other tissues within the insect by the BV (Figure 3A). This was recently demonstrated by following the progression of disease from a GP64-null AcMNPV baculovirus, a virus that does not produce infectious BV (Monsma *et al*., 1996). The GP64-null virus is also defective for cell-to-cell movement in cell culture. Thus, the role of the BV is the dissemination of infection from cell to cell and from tissue to tissue within the infected animal. The typical infection is believed to initiate by a round of viral replication in the midgut epithelium, and the subsequent production of progeny BV by budding from the basal side of midgut epithelial cells. In addition, an alternative mechanism for production of BV has also been demonstrated. When lepidopteran larvae are experimentally infected by feeding high doses of ODV, BV can be observed budding from the basal side of MG epithelial cells and infectious virus can be detected in the hemolymph within a few hours (Granados & Lawler, 1981). Thus, Granados & Lawler (1981) proposed that an alternative pathway of viral pathogenesis may involve the rapid conversion of ODV to BV, prior to viral replication in the midgut. The observation that BV can be produced prior to viral DNA replication is consistent with the early expression of the major envelope glycoprotein, GP64 EFP. Unlike most structural protein genes which are regulated as late transcription units, GP64 EFP is expressed both early and late in the infection cycle (Blissard & Rohrmann, 1989; Blissard & Rohrmann, 1991; Bradford *et al*., 1990; Jarvis & Garcia, 1994; Whitford *et al*., 1989). Because infected midgut epithelial cells may be rapidly shed in some larvae, a mechanism for rapidly traversing this tissue may provide a selective advantage. In addition, such a mechanism would require that multiple nucleocapsids enter a single cell so that some nucleocapsids may uncoat and express GP64 EFP (and any other necessary gene products), while other nucleocapsids migrate to the basal side of the cell for subsequent budding. Thus, two mechanisms for traversing or penetrating the midgut epithelial cell layer appear to be possible in baculovirus infections: One, perhaps the typical mechanism, requires DNA replication for amplification of virus in the midgut cell. The second mechanism (direct budding without viral replication) would not provide the benefit of viral amplification in the midgut but would rapidly move the virus through the midgut epithelial cell. How does the BV traverses the basal lamina of the midgut epithelium to infect tissues within the hemocoel? One possibility is that BV may directly traverse the basal lamina of the midgut epithelium during budding (Granados & Lawler, 1981). However, recent studies (Engelhard *et al*., 1994; Flipsen, 1995) using recombinant "marker" viruses to follow the sequence of tissues infected, suggest that the BV may also use the tracheal system as a conduit to cross the basal lamina of the midgut epithelium, since tracheoblasts are among the first cells infected after midgut epithelial cells. Thus, in the animal, infection begins by ODV infection of the midgut and production of infectious BV, which disseminates the infection first through the tracheal cells and perhaps hemocytes, and then to other tissues within the hemocoel.

Cellular entry by BV

In contrast to the ODV, which enter cells by direct fusion at the plasma membrane, BV enter cells by endocytosis (Figure 3). Entry by endocytosis was originally demonstrated by showing that BV infectivity

was neutralized by chloroquine and ammonium chloride (reagents that buffer the endosomal pH) (Volkman & Goldsmith, 1985). Viral entry by endocytosis is a multistep process that usually includes: 1) virion binding to a host cell receptor, 2) invagination of the host plasma membrane, 3) formation of an endocytic vesicle containing the enveloped virion, 4) acidification of the endosome, 5) activation of the viral envelope fusion protein, 6) fusion of the viral and endosomal membranes, and 7) release of the viral nucleocapsid into the cytoplasm (Figure 3). While a specific virus-cell receptor interaction has not been characterized for the baculovirus BV, scatchard analysis of BV interactions with insect cells indicate that specific binding between the BV and a host cell ligand occurs (Wickham *et al*., 1990). Data from the envelope protein of an unrelated virus may provide some insight into the identity of the baculovirus protein involved. Morse and coworkers (Morse *et at*., 1992) found that the envelope glycoproteins of the Thogoto (THO) and Dhori (DHO) viruses (orthomyxovirus-like arboviruses that are vectored by ticks) contain a remarkable degree of amino acid sequence identity with the baculovirus GP64 EFP protein, indicating a clear but unexplained ancestral relationship between the envelope protein genes of these two unrelated virus groups. Studies of the THO envelope protein have demonstrated both fusion and hemagglutinating activities (Portela *et al*., 1992). Hemagglutination serves as an indicator of receptor binding activity. Because of the high level (and colinearity) of sequence identity between the baculovirus GP64 EFP and the THO envelope protein, similarities in function are likely. Indeed, membrane fusion activity has been demonstrated for the baculovirus GP64 EFP Blissard & Wenz, 1992; Monsma & Blissard, 1995) but host receptor binding activity has not. Thus, while the baculovirus GP64 EFP protein appears to be a likely candidate BV "attachment protein," data to demonstrate this function are lacking, and the possibility that BV attachment or binding activity may reside in a different BV envelope protein cannot be excluded.

After BV binding at the plasma membrane and uptake of the virion into an endocytic vesicle, membrane fusion must occur before the nucleocapsid can be released into the cytoplasm. The fusion of biological membranes is a multistep process that is not well understood, even in the most intensively studied systems. [For discussions of membrane fusion models, the reader is referred to the following reviews: (Benz, 1993; White, 1992; Zimmerberg *et al*., 1993)]. In the case of the baculovirus BV, membrane fusion and nucleocapsid release likely involves the following processes: a) docking of the BV envelope and host endosome membrane (possibly mediated by the same interaction required for BV binding to the cell), b) triggering of fusion activity by low pH, c) merger of the outer membrane leaflet and membrane mixing, d) merger of the inner leaflet to form a fusion pore, and e) expansion of the fusion pore (Figure 3B). Membrane fusion of the BV envelope and endosome membrane is mediated by GP64 EFP. Initial studies utilizing a neutralizing monoclonal antibody (AcV1) directed against GP64 EFP showed that the GP64 EFP protein was necessary for the acid-induced fusion activity of the purified BV (Hohmann & Faulkner, 1983; Volkman & Goldsmith, 1985). More recent studies showed that GP64 EFP expressed on the surface of uninfected insect cells (in the absence of other viral proteins) was sufficient to mediate acid-induced membrane fusion activity (Blissard & Wenz, 1992; Monsma & Blissard, 1995). Thus, the GP64 EFP is necessary and sufficient for the acid-induced membrane fusion activity that is required for fusion of the BV envelope and endosome membrane.

GP64 EFP is extensively processed and is one of the best characterized of baculovirus proteins. GP64 EFP is glycosylated, phosphorylated, acylated, and contains intra- and inter-molecular disulfide bonds (Hohmann & Faulkner, 1983; Jarvis & Finn, 1995; Jarvis & Garcia, 1994; Oomens *et al*., 1995; Roberts & Manning, 1993; Roberts & Faulkner, 1989; Volkman, 1986; Volkman & Goldsmith, 1984). The native protein is present on the cell surface and in the BV envelope as an oligomer. Recent mass spectrometry analysis, using a soluble form of GP64 EFP, indicates that the oligomeric form of GP64 EFP is trimeric (Oomens *et al*., 1995). In addition, a recent mutagenesis study of GP64 EFP showed that a predicted amphipathic alpha helical domain containing a leucine zipper motif, is necessary for oligomerization (Monsma & Blissard, 1995).

How does GP64 EFP participate in the fusion process? In the best characterized membrane fusion protein, hemagglutinin (HA) of influenza virus, structural studies indicate that exposure to acid pH in the endosome results in a conformational change that propels the hydrophobic N-terminal domain of HA_2 (the fusion peptide) into the adjacent (endosomal) membrane (Carr & Kim, 1993). Although the baculovirus GP64 EFP bears no apparent similarity to the influenza HA protein, a similar displacement or exposure of a hydrophobic domain may be necessary. Unlike HA, which is proteolytically processed to produce a rela-

lively large (approximately 20 amino acids) hydrophobic N-terminal fusion peptide, the baculovirus GP64 EFP is not similarly processed and contains no terminal hydrophobic domain on the mature protein (Monsma & Blissard, unpublished). In the highly conserved GP64 EFP proteins of AcMNPV and OpMNPV, the most highly hydrophobic portion of the ectodomain consists of a relatively small, 6 amino acid region near the center of the protein. Using amino acid substitution mutations in the OpMNPV GP64 EFP protein, it was recently demonstrated that this small hydrophobic domain was required for membrane fusion activity (Monsma & Blissard, 1995). Whether this fusion domain is involved in the triggering of fusion, membrane mixing, or pore formation remains to be determined.

The use of cell-to-cell fusion, mediated by GP64 EFP expressed on the surface of infected cells, has served as a valuable technique for studying mechanisms by which GP64 EFP mediates membrane fusion within the endosome. Using AcMNPV infected Sf9 cells for cell-to-cell fusion studies, it has been shown that two of the steps in membrane fusion, triggering and membrane mixing, can be experimentally separated. By performing membrane fusion studies in the presence or absence of a lysolipid (lysophosphatidylcholine), it was demonstrated that lysophosphatidylcholine inhibits GP64 EFP mediated fusion of infected cells at a step after triggering, but prior to membrane merger (Chernomordik *et al*., 1995b; Chernomordik *et al*., 1993; Vogel *et al*., 1993) (see Figure 3B). Although the mechanism of lysolipid inhibition is not clear, it is believed that when inserted into membranes, the molecular shape of lysophosphatidylcholine (an inverted cone) may alter the ability of membranes to bend into the highly curved membrane intermediates that are necessary for membrane fusion to proceed (Chernomordik *et al*., 1995a; Chernomordik *et al*., 1995b; Zimmerberg *et al*., 1993). It is clear that this specific inhibitor will be useful for dissecting the steps in GP64 EFP-mediated membrane fusion and will provide a powerful tool for understanding the molecular and biophysical interactions between viral and host membranes.

Nucleocapsid transport and uncoating

After baculovirus nucleocapsids enter the cytoplasm, they are transported to the nucleus. Although little is known about this process, host cell actin cables may play a role in these early stages of infection. Actin is an abundant cellular protein that is involved in numerous cellular activities (cell movement, phagocytosis, secretion, etc.) and cell structure. In studies of BV entry into cultured Sf21 cells, Charlton & Volkman (1993) observed that filamentous actin (F-actin) aggregates within 30 minutes post infection. The formation of F-actin aggregates can be inhibited by treatments that prevent virion entry but not by cyclohexamide, suggesting that nucleocapsid entry, rather than production of early viral proteins results in F-actin cable formation. Surprisingly, cytochalasin D, a fungal toxin that binds to actin and prevents polymerization, does not appear to affect the efficiency of infection by BV (Volkman *et al*., 1987). Thus, although significant changes in the localization of F-actin result from viral entry into host cells, the role of actin during the early stages of infection is unknown.

Uncoating of baculovirus DNA occurs at or within the nucleus. Electron microscopic studies indicate that the two baculovirus genera, the NPVs and GVs, differ in the location of viral DNA uncoating. While nucleocapsids of NPVs enter host cell nuclei and uncoat within the nucleus (Granados, 1978), GV nucleocapsids appear to remain in the cytoplasm and line up at the nuclear pore, releasing viral DNA directly into the nucleus through the pore (Summers, 1971). Baculovirus nucleocapsids are cylindrical structures composed of helically wound subunits of the capsid protein. Optical diffraction studies suggested that each turn of the helix may consist of 12 copies of the capsid protein (Burley *et al*., 1982). The ends of the capsid are morphologically dissimilar to the cylindrical portion of the capsid and have been described as "nipple and claw" structures (Figure 2). One end, the "nipple" end, has the appearance of stacked rings of decreasing diameter (Federici, 1986; Teakle, 1969). One might speculate that specific structures or proteins at the end of the baculovirus nucleocapsid may interact with components of the nuclear pore complex. One viral protein, the P78/83 phosphorylated capsid protein, appears to be localized at one end of the nucleocapsid (Pham & Sivasubramanian, 1992; Possee *et al*., 1991; Vialard & Richardson, 1993) and thus may be a candidate for possible interactions with nuclear pores.

The extrusion of viral DNA from the intact nucleocapsid is believed to involve the phosphorylation of the basic DNA binding protein (known also as Basic Protein, P6.9, or VP12) (Russell & Rohrmann, 1990; Wilson *et al*., 1987). The basic DNA binding protein is associated with viral DNA in the nucleocapsid. In addition, a protein kinase activity capable of

phosphorylating the Basic DNA Binding Protein has been identified from nucleocapsids of the Plodia interpunctella GV (PiGV) (Wilson & Consigli, 1985a; Wilson & Consigli, 1985b). Although the Basic DNA Binding Protein is phosphorylated in infected cells, it is not phosphorylated in mature nucleocapsids and is associated with zinc in the nucleocapsid. Interestingly, Zn^{2+} was found to inhibit the nucleocapsid-associated kinase activity. In vitro studies have shown that activation of the nucleocapsid associated kinase (by a divalent cation, Mn^{2+} or Mg^{2+}) results in extrusion of viral DNA, similar to the uncoating observed in natural infections (Wilson & Consigli, 1985b). It has been noted (Funk & Consigli, 1993) that the basic DNA binding protein has similarities to cellular protamines, the simple and highly basic proteins that substitute for histones in the packaging of DNA within sperm of many species. Similarities between the basic DNA binding protein and protamines include the highly basic charge that results from a high arginine content, the ability to bind zinc and the cycling of phosphates. Funk & Consigli (1993) proposed the following model for uncoating of baculovirus DNA. The stable nucleocapsid contains the unphosphorylated form of the Basic DNA Binding Protein which is also complexed with zinc. At the time of uncoating, the Zn^{2+} may be chelated, activating the nucleocapsid associated kinase which then phosphorylates the Basic DNA Binding Protein. As with protamines, the phosphorylated form of the Basic DNA Binding Protein may have a lower affinity for DNA, resulting in unwrapping and the extrusion of DNA from the capsid. While a number of aspects of this model are speculative, the structural and functional similarities between eukaryotic protamines and the basic DNA binding protein make this an attractive model for the packaging and uncoating of viral DNA.

Early gene expression

Upon uncoating in the nucleus, unreplicated viral DNA is transcribed by a host RNA polymerase. Because early viral transcription is inhibited by alpha amanitin, a fungal toxin that specifically inhibits eukaryotic RNA polymerase II, host RNA polymerase II is believed to mediate most, if not all, early transcription from the viral genome (Fuchs *et al*., 1983). Although transcription from only a few baculovirus early genes has been examined in detail, promoter sequences from baculovirus early genes resemble insect RNA polymerase II promoters.

Core promoter elements

In many early promoters, a canonical TATA box is found approximately 30 bp upstream of the transcription start site. The role of the TATA box in eukaryotic RNA polymerase II promoters is well defined as a site for recognition and binding of the "TATA binding protein" (TBP). TBP binding to TATA sequences nucleates the assembly of an RNA polymerase II complex that directs transcription initiation to a site approximately 30 nt downstream. Experiments in which TATA boxes from baculovirus early promoters have been deleted or mutagenized confirm the function of these basal elements in baculovirus early promoters (Blissard *et al*., 1992; Blissard & Rohrmann, 1991; Dickson & Friesen, 1991; Guarino & Smith, 1992; Kogan *et al*., 1995; Pullen & Friesen, 1995b; Theilmann & Stewart, 1991). In addition, basal promoter elements such as the TATA box are functionally reiterated in some (perhaps many) baculovirus early genes. In one case, this basal promoter redundancy takes the form of dual TATA boxes (Guarino & Smith, 1992), while in other cases, overlapping TATA-dependent and TATA-independent basal promoter activities may provide basal promoter redundancy (Kogan *et al*., 1995; Pullen & Friesen, 1995b). TATA-less baculovirus early promoters have also been identified but have not been studied as extensively as TATA-containing promoters.

Baculovirus early genes frequently contain a conserved "CAGT" sequence at the transcription start site and are similar in this regard to insect RNA polymerase II genes (Blissard & Rohrmann, 1989; Blissard & Rohrmann, 1990; Bucher, 1990; Cherbas & Cherbas, 1993; Hultmark *et al*., 1986). Conservation of sequences at the transcription start site is not similarly observed in vertebrate RNA polymerase II genes (Bucher, 1990). In the majority of cases where baculovirus early transcription has been mapped within or near a CAGT sequence, a TATA box is also located upstream. Although a survey of the AcMNPV genome indicates that the conserved CAGT motif is located upstream of many of the predicted ORFs (Ayres *et al*., 1994), an understanding of the overall distribution of these motifs and associations with early promoters will require extensive transcriptional mapping of the genome. Functionally, the conserved CAGT start site sequence has been shown to play an important role in the efficiency of transcription initiation in several baculovirus early promoters (Blissard *et al*., 1992; Guarino & Smith, 1992; Kogan *et al*., 1995; Pullen & Friesen, 1995b). In some cases, sequences at or near the start

site (including the start site CAGT) may be essential for core or basal promoter activity although a strict conservation of the CAGT sequence may not be required (Kogan *et al.*, 1995; Pullen & Friesen, 1995a; Pullen & Friesen, 1995b). It is currently unclear whether the conserved CAGT start site sequence represents a recognition sequence for host transcription factor binding, or an optimal sequence for protein-DNA contacts at the initiation site of an RNA polymerase II complex that was previously bound at another site. Studies examining general transcription factor IID (TFTID) interactions with core elements of RNA polymerase II promoters from *Drosophila melanogaster* indicate that sequences at the transcription start site influence binding of the TFIID complex. Selection of random start site sequences by binding to Drosophila TFIID identified a consensus sequence (G/T/A T/*C A G/T T* G) (Purnell *et al.*, 1994) that correlates well with the CAGT identified at the transcription start sites of baculovirus genes. Thus, the conservation of the start site CAGT sequence in early baculovirus promoters may promote or stabilize the binding of the host TFIID complex.

Host modulation of early transcription

In addition to studies of basal transcription, a number of studies have used promoter mutagenesis in the context of either transient expression assays or recombinant baculoviruses, to identify sequence elements that modulate the activity of baculovirus early promoters. Few consensus sequences have emerged. Using electrophoretic mobility shift analysis (EMSA) to examine the physical interaction of host proteins with early promoters, it was shown that at least two sequence motifs, $^{T}/_{A}$**GATA**$^{A}/_{G}$ (GATA elements) and CACGTG, are recognized and bound by host factors (Kogan & Blissard, 1994; Krappa *et al.*, 1992). In one case, host factor binding to these motifs was shown to activate RNA polymerase II mediated transcription in transient expression assays (Kogan & Blissard, 1994). Each of these motifs represents a sequence recognized by a large family of eukaryotic transcription factors: GATA factors and "basic region/helix-loop-helix/leucine zipper" (B-HLH-Zip) proteins (Giacca *et al.*, 1992; Orkin, 1992). The utilization of sequence motifs recognized by large families of eukaryotic transcription factors may represent a viral strategy for insuring activation of early genes in the widest possible range of host tissues and cells. These binding sites may also be multifunctional, contributing to promoter element redundancy. In addition to their abilities to serve as activating elements in a promoter containing a functional TATA box, both GATA and CACGTG binding sites were also shown to serve as components of a core promoter in a TATA-less context (Kogan *et al.*, 1995). A similar multifunctionality of upstream promoter elements (and redundancy in roles) has been reported in the Adenovirus major late promoter (Reach *et al.*, 1991). The 5′ untranslated regions (5′ UTR) of some baculovirus early genes also contain transcriptional regulatory elements that may serve as core promoter elements or activating elements (Kogan *et al.*, 1995; Pullen & Friesen, 1995b).

Viral modulation of early transcription

Baculoviruses encode at least 4 gene products that appear to regulate transcription from viral early genes. The IE0, IE1, IE2 (IEN), and PE38/P34 proteins have been identified by transient expression assays as baculovirus gene products regulating early promoters (Carson *et al.*, 1988; Guarino & Summers, 1986a; Guarino & Summers, 1987; Krappa & Knebel, 1991; Lu & Carstens, 1993; Theilmann & Stewart, 1991; Theilmann & Stewart, 1992; Wu *et al.*, 1993; Yoo & Guarino, 1994). The best studied of these transcriptional transactivators, IE1, activates transcription from numerous early promoters and may function by both sequence-dependent and sequence-independent mechanisms. While sequence-independent activation is poorly understood, sequence-dependent activation by IE1 has been identified and studied by EMSA, transient expression assays, and in recombinant viruses. In vitro studies of IE1 binding to viral DNA show that the IE1 protein forms complexes with repeated sequences known as homologous region (hr) sequences (Choi & Guarino, 1995a; Choi & Guarino, 1995b; Guarino & Dong, 1994; Kovacs *et al.*, 1992; Leisy *et al.*, 1995; Rodems & Friesen, 1995). The hr sequences in the AcMNPV genome are so named because they represent homologous (or similar) sequences found at eight locations distributed around the circular genome (Ayres *et al.*, 1994; Cochran & Faulkner, 1983) (Figure 1A, hr). Studies of hr sequences revealed that at each location, the hr consists of a series of 30 bp imperfect palindromes, with each palindrome separated from the next by approximately 50–115 bp (Guarino *et al.*, 1986). Since each imperfect palindrome contains an EcoRI site at its center, each hr site contains multiple EcoRI sites (Figure IB). Functional studies have demonstrated that hrs serve as both enhancers of early

transcription (Guarino *et al*., 1986; Guarino & Summers, 1986b; Rodems & Friesen, 1993) and as putative origins of DNA replication (Kool *et al*., 1993a; Kool & Vlak, 1993; Kool *et al*., 1993b; Leisy & Rohrmann, 1993; Pearson *et al*., 1992). Using truncated IE1 proteins for binding to hr sequences (in EMSA experiments and transient expression assays), the N-terminal region of IE1 was tentatively identified as an acidic activation domain, while DNA binding activity was assigned to the more C-terminal region (Kovacs *et al*., 1992).

One cellular process that is uncommon for baculovirus mRNAs, is RNA splicing. In some dsDNA viruses that are transcribed in the nucleus (such as Adenoviruses and Papovaviruses), viral RNAs are spliced and alternative splicing plays an important role in the regulation of gene expression. In another group of nuclear dsDNA viruses, the Herpesviruses, few mRNAs are derived by splicing. Splicing has been detected in the RNA of only one baculovirus gene, *ie0* (Chisholm & Henner, 1988; Kovacs *et al*., 1991a). The *ie0* mRNA is produced by splicing a single intron from a long primary transcript that contains a small upstream exon and a large downstream exon that contains the majority of the *iel* ORE The *ie0* splicing event results in an mRNA that encodes a predicted protein very similar to the IE1 protein, but with 54 additional N-terminal amino acids. Like *iel*, *ie0* RNAs are produced both early and late in the infection cycle, although *ie0* RNAs appear to initiate at different sites during the late phase (Kovacs *et al*., 199la). Transient expression assays indicate that the IE0 protein also serves as a transactivator of transcription, but the activities of IE0 differ somewhat from those of IE1 (Kovacs *et al*., 1991b).

Early to late phase transition

The transition from the early to late phase of the baculovirus infection cycle is characterized by replication of viral DNA, activation of an alpha-amanitin resistant DNA-dependent RNA polymerase activity, and the apparent inhibition of host transcription. The replication of viral DNA appears to be a prerequisite for late transcription since aphidicolin, an inhibitor of viral (and host) DNA polymerase activity, also inhibits baculovirus late transcription (Miller *et al*., 1981; Rice & Miller, 1986). Concomitant with viral DNA replication, levels of host mRNAs also decline substantially, suggesting that host transcription is inhibited (Ooi & Miller, 1988). Whether host translation is specifically inhibited by the virus is not known. In *Spodoptera frugiperda* cells infected with AcMNPV, host protein synthesis begins to decrease around 6–10 hours post infection and appears to be completely absent by 24 hours post infection (Carstens *et al*., 1979; Wood, 1980). Virus-host interactions at the translational level may directly or indirectly affect the ability of a baculovirus to replicate in a given cell. In cell culture, translational inhibition has been observed in non-productive infections. When two non-permissive *Lymantria dispar* cell lines were infected with AcMNPV, both early and late viral transcription appeared normal but viral and host protein synthesis were inhibited completely during the late phase, suggesting that the block in successful viral replication may be at the translational level (Guzo *et al*., 1992). More recently, a block in viral replication was examined in another virus-host system. The Bombyx mori NPV (BmNPV) and AcMNPV baculoviruses are very closely related (approximately 95% identical in nucleotide sequence; (Majima *et al*., 1993)) but they exhibit distinct host range differences in nature and in cell culture. Using recombination between these closely related viruses, a single gene that modulates the host range restriction was identified (Croizier *et al*., 1994; Kamita & Maeda, 1993; Maeda *et al*., 1993). This baculovirus "host range gene" is known as *p143* (or *helicase),* a gene that was previously reported to be involved in DNA replication and to contain seven motifs (near the C-terminus of the 1221 amino acid protein) common to proteins involved in DNA helicase activity (Lu & Carstens, 1991). In addition, the *p143* gene has also been identified as essential for both DNA replication (Kool *et al*., 1994) and late gene expression (Passarelli & Miller, 1993) in transient replication and expression assays, respectively. A single amino acid change (Valine to Methionine at amino acid position 934), near one of the seven helicase motifs was previously reported to result in a temperature sensitive mutation (ts8) in viral DNA replication (Brown *et al*., 1979; Lu & Carstens, 1991). In contrast, the "host range" modifications in *p143* were mapped to three amino acids nearer the N-terminus of the protein (amino acids 556, 564, and 577). Similar to observations in the non-permissive *Lymantria dispar* cells (Guzo *et al*., 1992), it was reported that infection of non-permissive BmN cells with AcMNPV resulted in the attenuation of host and viral protein synthesis (Kamita & Maeda, 1993). However, infection of BmN cells with a recombinant AcMNPV virus containing a chimeric *p143* gene (con-

taining BmNPV amino acids from 413 to 602, and the remainder from AcMNPV) restored normal protein synthesis and viral replication. Additionally, coinfection experiments suggest that when expressed in an incompatible host cell line, the *p143* gene product is responsible for a dominant inhibition of translation (Kamita & Maeda, 1993). Whether the attenuation of viral protein synthesis in non-permissive hosts is the cause of host range restriction, or an indirect effect, remains to be determined. However, it appears that P143 is an important factor in the determination of host range in baculoviruses.

Late gene expression

The late phase of infection is marked by the appearance of an alpha amanitin resistant RNA polymerase activity (Grula *et al*., 1981) and the transcription of late genes. Although little is currently known about the biochemical composition of the baculovirus late RNA polymerase (Xu *et al*., 1995; Yang *et al*., 1991), nuclear extracts from baculovirus infected cells are capable of supporting accurate transcription from late promoters (Glocker *et al*., 1993). Thus fractionation of nuclear extracts and purification and biochemical characterization of the late RNA polymerase should be possible. A number of viral gene products required for late transcription have been identified by transient expression studies. To date, at least 18 baculovirus genes have been identified as *late expression factor* genes or "*lef*" genes (Todd *et al*., 1995). Of the 18 *lef* genes, 9 are involved in DNA replication, while 10 appear to affect late transcription more directly (Kool *et al*., 1994; Lu & Miller, 1995). Specific biochemical functions have been assigned to some *lef* genes and possible biochemical roles have been hypothesized for others (Ahrens *et al*., 1995; Hang *et al*., 1995; Lu & Miller, 1995; Todd *et al*., 1995). In addition to the *lef* genes, one viral gene that is specifically required for the high level very late gene expression (exhibited by the *polyhedrin and p10* genes) has been identified and named "*very late factor–1*" or *vlf–1* (McLachlin & Miller, 1994). While early transcription is believed to be mediated primarily by the general host transcription factors that assemble to form a host RNA polymerase II complex, the role(s) of host proteins in late transcription are unknown. A host phosphoprotein of approximately 30 kDa that binds with high affinity to the AcMNPV polyhedrin promoter was recently identified (Burma *et al*., 1994). However, the functional role of this host protein in baculovirus late or very late transcription remains to be determined.

With only rare exceptions, baculovirus late transcription initiates within a conserved TAAG sequence found at the transcription start site. This conserved start site sequence comprises the core of the baculovirus late promoter. In late promoters that have been studied, the conserved, almost invariant, core "TAAG" sequence is usually preceded by an A, (and less frequently by a T or G) nucleotide. A mutational analysis of the late promoter from the AcMNPV capsid protein gene (*vp39*) showed that only sequences within 6–8 nt adjacent to the core TAAG motif significantly affected late transcription (Morris & Miller, 1994). In addition, an 18 bp sequence containing the TAAG core at its center was transcriptionally active in infected cells. Similar studies of the AcMNPV polyhedrin gene (a very late gene) have identified an 8 bp (TAAG-containing) sequence at the transcription start site as the primary determinant of transcription (Ooi *et al*., 1989; Rankin *et al*., 1988). Because of their extremely high levels of transcription, the *polyhedrin* and *p10* genes have been described as "hyper-expressed" late genes. A comparison of polyhedrin gene promoters shows a conservation of AT rich sequences immediately downstream of the TAAG core sequence (Rohrmann, 1986). Mutational analyses indicate that sequences immediately upstream and downstream of the polyhedrin TAAG are important for the exceptionally high levels of transcription (Ooi *et al*., 1989).

Viral assembly

Following synthesis of late gene products, nucleocapsid assembly begins within the nucleus. While little is known about nucleocapsid assembly at the biochemical and molecular level, electron micrographs indicate that empty capsids assemble within nuclei, in association with an electron dense "virogenic stroma" (Fraser, 1986b; Young *et al*., 1993). By immunofluorescent staining, nuclear F-actin appears to co-localize with the capsid protein (P39) in the nucleus, suggesting that actin may play a role in the assembly of nucleocapsids (Charlton & Volkman, 1991). Empty nucleocapsids, or nucleocapsids that appear to be in the process of packaging viral DNA, are often observed with one end in association with the virogenic stroma suggesting that capsids are first assembled, then "filled" with DNA (Fraser, 1986b). Packaging of viral DNA may involve the dephosphorylation of the protamine-like

basic DNA binding protein (P6.9) as only the non-phosphorylated form of P6.9 is found associated with mature nucleocapsids (Tweeten *et al*., 1980a; Tweeten *et al*., 1980b) (see previous discussion). Nothing is known regarding the mechanism that determines whether nucleocapsids migrate to the plasma membrane (for budding and production of BV) or remain within the nucleus for subsequent envelopment and occlusion there.

The very late phase of infection is characterized by the reduction or cessation of transcription from many late genes, and the so-called hyper-expression of very late genes such as the occlusion body protein (Polyhedrin) (Hooft *et al*., 1983) and a protein involved in the occlusion process, P10 (Kuzio *et al*., 1984; Leisy *et al*., 1986; Van-Oers *et al*., 1994; Zuidema *et al*., 1993). In the very late phase, the occlusion body protein associates with, and subsequently crystallizes around enveloped virions within the nucleus. This occlusion process appears to be completed by the addition of a protein-carbohydrate "envelope structure" (containing the polyhedral envelope protein; PEP, or PP34) around the occlusion body (Gombart *et al*., 1989; Rohrmann, 1992; Whitt & Manning, 1988) (Figure 2). The maturation and release of occlusion bodies from infected cells requires the very late protein P10. Very late in infection, fibrillar structures that contain P10 form in both cytoplasm and nucleus. Deletion of the P10 gene, while not lethal, results in several major effects: defective addition of the "envelope" to the occlusion body, impaired nuclear disintegration, and defective cell lysis (Van-Oers *et al*., 1993; Van-Oers *et al*., 1994; Williams *et al*., 1989). Thus, in AcMNPV viruses lacking a functional P10, polyhedra are not released from infected cells by normal cell lysis, and polyhedra produced from these viruses are fragile and sensitive to disruption by physical stress.

Molecular and cellular responses to infection

At the molecular level, very little is known regarding the variety of responses of the insect cell to invasion by baculoviruses. However, in the past few years discoveries of viral responses to host cell defenses have provided a fascinating picture of the virus-cell interplay. One cellular response that has received considerable recent attention is apoptosis. Apoptosis, or "Programmed Cell Death," is a mechanism that multicellular organisms utilize to regulate development of tissues, and to eliminate damaged or diseased cells. In diseased or damaged cells, apoptosis is often associated with cellular detection of either metabolic disturbances, single stranded DNA, or damaged DNA. The characteristic symptoms of apoptosis include cellular shrinkage, chromatin condensation, nuclear fragmentation, chromosomal DNA degradation into oligonucleosomal length fragments, and extensive "blebbing" and pinching of vesicles into the medium. Upon infection by viruses, many cell types appear to be capable of inducing a cascade leading to apoptosis, as a defensive measure (Vaux *et al.,* 1994). In many cases, viral invasion of mammalian cells results in the induction of *p53*, the tumor suppressor gene that appears to induce or facilitate apoptosis. As an "evolutionary response" to host cell apoptosis, many viruses carry genes that encode inhibitors of apoptosis. Viruses known to encode functional inhibitors of apoptosis include Adenovirus, SV40, Papillomavirus, Cowpox Virus, and Baculovirus. In some cases, viral inhibitors of apoptosis are known to function by directly inactivating a cellular protein that induces apoptosis. Adenovirus E1B55kD and SV40 large T antigen are examples of viral proteins that functionally inactivate mammalian P53. Viral proteins may also inhibit apoptosis by binding to and inactivating proteins within the cell death pathway. The Cowpox virus *crmA* gene product has been shown to bind and inactivate Interleukin–1 Beta Converting Enzyme (ICE), a protein in the apoptosis cascade. In yet other cases, viral inhibitors of apoptosis resemble normal cellular inhibitors of apoptosis. Epstein-Barr Virus and African Swine Fever Virus encode proteins that resemble the *B-cell lymphoma–2* gene product (BCL–2), a (mammalian) cellular inhibitor of apoptosis.

The induction of apoptosis in baculovirus-infected insect cells was first observed in insect Sf21 cells infected with an AcMNPV virus lacking a functional *p35* gene (Clem *et al*., 1991). In this case, induction of apoptosis appears to be cell-type specific, as similar effects were not observed in TN–368 cells. Subsequent studies confirmed that the baculovirus P35 protein inhibits apoptosis in baculovirus infected cells (Clem *et al*., 1991; Clem & Miller, 1993; Clem & Miller, 1994a; Clem & Miller, 1994b; Hershberger *et al*., 1992) and will also inhibit programmed cell death in *Drosophila melanogaster*, *Caenorhabditis elegans*, and mammalian cell lines (Beidler *et al*., 1995; Hay *et al*., 1994; Rabizadeh *et al*., 1993; Sugimoto *et al*., 1994). Although baculovirus P35 will inhibit apoptosis in these heterologous systems, the mammalian *bcl–2* gene product cannot substitute for P35 in bac-

ulovirus infected insect cells (Cartier *et al*., 1994; Clem & Miller, 1994a).

In addition to P35, another inhibitor of apoptosis has been identified in two additional baculoviruses. Genes capable of functionally complementing *p35*$^{(-)}$ AcMNPV viruses were identified from the genomes of OpMNPV and Cydia pomonella Granulosis Virus (CpGV) (Birnbaum *et al*., 1994; Crook *et al*., 1993). These functional homologs of P35 are known as "Inhibitor of Apoptosis" or IAP proteins and were named according to the virus from which they were identified: Op-IAP and Cp-IAP. The AcMNPV genome also contains two genes with similarities to the Op-IAP and Cp-IAP (Figure 1, *iap1* and *iap2*) but these AcMNPV genes cannot functionally substitute for *p35* in Sf21 cells.. While the AcMNPV P35 protein shows no sequence similarity to any known regulator of apoptosis, the Op-IAP and Cp-IAP proteins show significant amino acid sequence similarity to a neuronal apoptosis inhibitory protein (NAIP) which is believed to regulate apoptosis in mammalian neurons (Roy *et al*., 1995). Thus, baculoviruses encode at least two gene products capable of inhibiting host cell apoptosis.

Interactions of host and viral DNA

A number of examples of host cell DNA insertions into the baculovirus genome have been documented and several reviews have addressed this topic in detail (Blissard & Rohrmann, 1990; Fraser, 1986a; Friesen, 1993). Host DNA insertions were first noted as the cause of "few polyhedra" or FP phenotype mutants that arose from repeated serial passage of viruses in cell culture. Most FP mutants were detected as either insertions of host transposable elements or deletions in the "FP locus" at approximately 36 map units of the AcMNPV genome (see Figure 1). The FP locus encodes the 25K gene, a gene involved in intranuclear envelopment of nucleocapsids and occlusion of ODV (Beames & Summers, 1988; Beames & Summers, 1989; Harrison & Summers, 1995a; Harrison & Summers, 1995b). The host DNA insertions detected in the FP locus are usually small and insert most frequently at a "TTAA" target site that is duplicated upon transposition (Beames & Summers, 1990; Cary *et al*., 1989; Fraser *et al*., 1995; Fraser *et al*., 1983; Wang *et al*., 1989). Host DNA insertions have also been documented at other locations in the AcMNPV genome and in other baculoviruses. Another well characterized example is the insertion of the lepidopteran retrotransposon "TED" at 86.7 m.u. in the AcMNPV genome (Friesen & Nissen, 1990; Miller & Miller, 1982). The TED retrotransposon is a member of the gypsy family of retrotransposons and was first discovered as a result of its insertion into the AcMNPV genome after repeated serial passage of AcMNPV in cultured *Trichoplusia ni* cells. Gypsy retrotransposons are very similar to provirus forms of retroviruses. The TED element is 7.5 kbp and contains three overlapping open reading frames that are flanked by 5´ and 3´ long terminal repeats (LTRs) (Friesen & Nissen, 1990; Friesen *et al*., 1986; Lerch & Friesen, 1992). Functional and comparative studies of TED encoded genes have demonstrated that two of the TED reading frames encode proteins analogous to the gag and pol gene products of retroviruses (Lerch & Friesen, 1992). A third example of host DNA insertions into a baculovirus genome comes from the apparent insertion of transposons into the genome of the *Cydia pomonella* Granulosis Virus (CpGV) during infection of an insect (Jehle *et al*., 1995). One insertion, TCI4.7 was acquired during a mixed infection of *Cryptophlebia leucotreata* larvae with both *C. leucotreata* GV (C1GV) and CpGV. TCI4.7 consists of a 4.7 kb insert that contains 29 bp inverted terminal repeats. In addition this apparent transposon contains an open reading frame with amino acid sequence similarities to transposases from Tc1-like transposable elements, suggesting that TCI4.7 may encode a transposase. Jehle and coworkers (Jehle *et al*., 1995) suggest that because TCI4.7 and other insertion sequences were detected by genotypic screening of viruses infecting whole animals, the movement of insect transposons into baculovirus genomes may be much more frequent in nature than would be expected from the use of phenotypic screening of viruses in cell lines. Thus, movement of host DNA into baculovirus genomes may provide a mechanism for viral acquisition of host genes, and may even facilitate the horizontal movement of insect genes between insect populations and species.

Other potentially interacting proteins

Several baculovirus genes encode proteins with amino acid sequence similarities to known eukaryotic proteins. These include proteins with known similarities to Cu/Zn superoxide dismutase (Tomalski *et al*., 1991), omega-conotoxin (Eldridge *et al*., 1992), and ubiquitin (Guarino, 1990; Russell & Rohrmann, 1993b). Although these viral gene products may be involved in

viral interactions with host cell components, functional roles in the infection cycle have not been defined. Two genes that encode proteins with predicted similarities to protein kinases have also been identified in the AcMNPV genome (Figure 1, *pk1* and *pk2*) (Ayres *et al.*, 1994). Although the pk1 gene product has protein kinase activity and is expressed late in infection, PK1 does not appear to have characteristics similar to the capsid associated kinase (Reilly & Guarino, 1994). Kinase activity has not been detected from the pk2 gene product (Li & Miller, 1995).

Whole animal considerations

In addition to intracellular interactions, baculoviruses also encode gene products that function at the organismal level and thus manipulate the physiology and structure of the infected animal. One gene product, Ecdysteroid UDP-glucosyltransferase (EGT) extends the larval stage by inactivating host ecdysteroids, the steroid hormones that regulate insect molting (O'Reilly, 1995; O'Reilly *et al.*, 1992a; O'Reilly & Miller, 1989; O'Reilly & Miller, 1990). Infected cells secrete the EGT enzyme into the hemolymph, where EGT catalyzes the transfer of galactose (or possibly glucose) to ecdysone, producing an inactive sugar conjugate of the hormone. Thus, EGT prolongs the larval stage of the insect host by preventing the accumulation of high litres of active ecdysone, a condition necessary for molting to the pupal stage.

Another possible extracellular activity of baculoviruses involves the production of enzymes that aid in the release of the occluded virus from insect larvae. After infection has spread throughout a larval lepidopteran, many infected cells may lyse, releasing occlusion bodies within the body cavity. However, the tough larval exoskeleton (composed of protein and chitin) provides a significant barrier that might prevent release of occlusion bodies. Enzymes that may aid in the degradation of the insect exoskeleton are encoded by two baculovirus genes: chitinase and v-cath. An AcMNPV gene encoding a functional chitinase enzyme, similar to bacterial chitinases, was recently identified and characterized (Ayres *et al.*, 1994; Hawtin *et al.*, 1995). In addition, a cysteine protease with characteristics of the mammalian lysosomal protease, cathepsin L, is encoded by the AcMNPV, BmNPV, and CfMNPV viruses (Hill *et al.*, 1992; Ohkaa *et al.*, 1994; Slack *et al.*, 1995) (Figure 1, *v-cath*). Infection of insects with recombinant viruses containing an inactivated *v-cath* gene results in insects with an altered appearance (after death), that fail to disintegrate normally. Thus, V-Cath appears to facilitate the release of occlusions from the animal after death. Because cathepsins are general cysteine proteinases and V-Cath is produced in wild type virus infections, the activity of V-Cath may be problematic in the expression or purification of some proteins from baculovirus expression systems. However, because v-cath is not an essential gene (Ohkaa *et al.*, 1994; Slack *et al.*, 1995), the use of v-cath$^{(-)}$ mutants may prove to be useful in some circumstances.

Summary

Baculovirus interactions with host cells range from the physical interactions that occur during viral binding and entry, to the complex and subtle mechanisms that regulate host gene expression and modify and regulate cellular and organismal physiology and defenses. Fundamental studies of baculovirus biochemistry and molecular biology have yielded many interesting and important discoveries on the mechanisms of these virus-host interactions. Information from such studies has also resulted in exciting new strategies for environmentally sound insect pest control, and in the development and improvement of a valuable eukaryotic expression vector system. In addition a number of important and valuable model biological systems have emerged from studies of baculoviruses. These include robust systems for studies of eukaryotic transcription, viral DNA replication, membrane fusion, and apoptosis. Because functions have been identified for only a small number of baculovirus genes, we can expect many exciting new discoveries in the future and an unfolding of the complex and intricate relationship between baculoviruses and insect cells.

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References

Ahrens, CH & Carlson C *et al.* (1995) Identification, sequence, and transcriptional analysis of *lef–3*, a gene essential for *Orgyia pseudotsugata* baculovirus DNA replication. Virology 210: 372–382.

Ayres MD & Howard SC *et al.* (1994) The complete DNA sequence of *Autographa californica* Nuclear Polyhedrosis Virus. Virology 202: 586–605.

Beames B & Summers MD (1988) Comparisons of host cell DNA insertions and altered transcription at the site of insertions in few polyhedra baculovirus mutants. Virology 162(1): 206–220.

Beames B & Summers MD (1989) Location and nucleotide sequence of the 25k protein missing from baculovirus few polyhedra FP mutants. Virology 168(2): 344–353.

Beames B & Summers MD (1990) Sequence comparison of cellular and viral copies of host cell DNA insertions found in Autographa californica Nuclear Polyhedrosis Virus. Virology 174: 354–363.

Beidler DR & Tewari M *et al.* (1995) The Baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. J Biol Chem 270(28): 16526–16528.

Benz J (ed.) (1993) Viral Fusion Mechanisms. CRC Press. Boca Raton.

Birnbaum MJ & Clem RJ *et al.* (1994) An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys-His sequence motifs. Journal of Virology 68(4): 2521–2528.

Blissard GW & Kogan PH *et al.* (1992) A synthetic early promoter from a baculovirus: Roles of the TATA box and conserved start site CAGT sequence in basal levels of transcription. Virology 190: 783–793.

Blissard GW & Rohrmann GF (1989) Location, sequence, transcriptional mapping, and temporal expression of the *gp64* envelope glycoprotein gene of the *Orgyia pseudotsugata* Multicapsid Nuclear Polyhedrosis Virus. Virology 170(2): 537–555.

Blissard GW & Rohrmann GF (1990) Baculovirus diversity and molecular biology. Annu. Rev. Entomol. 35: 127–155.

Blissard GW & Rohrmann GF (1991) Baculovirus *gp64* gene expression: Analysis of sequences modulating early transcription and transactivation by IE1. J. Virology 65: 5820–5827.

Blissard GW & Wenz JR (1992) Baculovirus GP64 envelope glycoprotein is sufficient to mediate pH dependent membrane fusion. J. Virology 66: 6829–6835.

Bradford MB & Blissard GW *et al.* (1990) Characterization of the infection cycle of the *Orgyia pseudotsugata* Multicapsid Nuclear Polyhedrosis Virus in *Lymantria dispar* cells. J Gen Virol 71(12): 2841–2846.

Braunagel SC & Elton DM *et al.* (1996) Identification and analysis of an *Autographa californica* Nuclear Polyhedrosis Virus structural protein of the occlusion-derived virus envelope: ODV-E56. Virology 217(1): 97–110.

Braunagel SC & Summers MD (1994) Autographa californica nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: Structural proteins, antigens, lipid and fatty acid profiles. Virology 202(1): 315–328.

Brown M & Crawford AM *et al.* (1979) Genetic analysis of a baculovirus, *Autographa californica* nuclear polyhedrosis virus. I. Isolation of temperature-sensitive mutants and assortment into complementation groups. J. Virol. 31: 190–198.

Bucher P (1990) Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. J Mol Biol 212(4): 563–578.

Burley SK & Miller A *et al.* (1982) Structure of the Baculovirus Nucleocapsid. Virology 120(2): 433–440.

Burma S & Mukherjee B *et al.* (1994) An unusual 30-kDa protein binding to the polyhedrin gene promoter of *Autographa californica* Nuclear Polyhedrosis Virus. Journal of Biological Chemistry 269(4): 2750–2757.

Carr CM & Kim PS (1993) A Spring-Loaded Mechanism For the Conformational Change of Influenza Hemagglutinin. Cell 73(4): 823–832.

Carson DD & Guarino LA *et al.* (1988) Functional mapping of an AcNPV immediate early gene which augments expression of the IE–1 trans-activated 39K gene. Virology 162(2): 444–451.

Carstens EB & Tjia ST *et al.* (1979) Infection of *Spodoptera frugiperda* cells wilh *Autographa californica* nuclear polyhedrosis virus. I. Synthesis of intracellular proteins after virus infection. Virology 99: 368–398.

Cartier JL & Hershberger PA *et al.* (1994) Suppression of apoptosis in insect cells stably transfected with baculovirus *p35*: Dominant interference by N-terminal sequences $p35^{1-76}$. J. Virology 68: 7728–7737.

Cary LC & Goebel M *et al. (*1989) Transposon mutagenesis of baculoviruses: Analysis of Trichoplusia-ni transposon IFP2 insertions within the fp-locus of nuclear polyhedrosis viruses. Virology 172(1): 156–169.

Charlton CA & Volkman LE (1991) Sequential rearrangement and nuclear polymerization of actin in baculovirus-infected *Spodoptera frugiperda* cells. J Virol 65(3): 1219–1227.

Charlton CA & Volkman LE (1993) Penetration of Autographa californica nuclear polyhedrosis virus nucleocapsids into IPLB Sf 21 cells induces actin cable formation. Virology. 197(1): 245–254.

Cherbas L & Cherbas P (1993) The arthropod initiator: the cap-site consensus plays an important role in transcription. Insect Biochem. Molec. Biol. 23(1): 81–90.

Chernomordik L & Kozlov MM *et al.* (1995a) Lipids in biological membrane fusion. J Membrane Biol 146(1): 1–14.

Chernomordik L & Leikina E *et al.* (1995b) Control of Baculovirus gp64-Induced Syncytium Formation by Membrane Lipid Composition. J. Virology 69(5): 3049–3058.

Chernomordik LV & Vogel SS *et al.* (1993) Lysolipids Reversibly Inhibit Calcium GTP and pH-Dependent Fusion of Biological Membranes. Febs Lett 318(1): 71–76.

Chisholm GE & Henner DJ (1988) Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE–1 gene. J. Virol. 62(9): 3193–3200.

Choi J & Guarino LA (1995a) Expression of the IE1 Transactivator of *Autographa californica* Nuclear Polyhedrosis Virus during Viral Infection. Virology 209: 99–107.

Choi J & Guarino LA (1995b) A temperature-sensitive IE1 protein of *Autographa californica* Nuclear Polyhedrosis Virus has altered transactivation and DNA binding activities. Virology 209: 90–98.

Clem RJ & Fechheimer M *et al.* (1991) Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science 254(5036): 1388–1390.

Clem RJ & Miller LK (1993) Apoptosis reduces both the in-vitro replication and the in-vivo infectivity of a baculovirus. J Virol 67(7): 3730–3738.

Clem RJ & Miller LK (1994a) Control of Programmed Cell Death by the Baculovirus Genes p35 and iap. Molecular and Cellular Biology 14(8): 5212–5222.

Clem RJ & Miller LK (1994b) Induction and inhibition of apoptosis by insect viruses. In: "Communications in Cell & Molecular Biology, Vol. 8. Apoptosis II: The molecular basis of apoptosis in disease.", Vol. 0, pp. 89–110. Cold Spring Harbor Laboratory Press, Plainview, New York, USA.

Cochran MA & Faulkner P (1983) Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. J. Virol. 45(3): 961–970.

Croizier G & Croizier L *et al.* (1994) Extension of Autographa-californica Nuclear Polyhedrosis Virus Host Range by Interspecific Replacement of a Short DNA Sequence in the p143 Helicase Gene. Proc Natl Acad Sci USA 91(1): 48–52.

Crook NE & Clem RJ *et al.* (1993) An Apoptosis-Inhibiting Baculovirus Gene With a Zinc Finger-Like Motif. J Virol 67(4): 2168–2174.

Derksen ACG & Granados RR (1988) Alteration of a Lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology 167: 242–250.

Dickson JA & Friesen PD (1991) Identification of upstream promoter elements mediating early transcription from the 35000-molecular-weight protein gene of *Autographa californica* nuclear polyhedrosis virus. J Virol 65(8): 4006–4016.

Eldridge R & Li Y *et al.* (1992) Characterization of a baculovirus gene encoding a small conotoxin- like polypeptide. J Virol 66(11): 6563–6571.

Engelhard EK & Kam-Morgan LNW *et al.* (1994) The insect tracheal system: A conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus. Proceedings of the National Academy of Sciences of the United States of America 91(8): 3224–3227.

Federici BA (1986) Ultrastructure of baculoviruses. In: "The Biology of Baculoviruses" RR Granados & BA Federici (eds.) Vol. I, pp. 61–88. 2 vols. CRC Press, Boca Raton, Florida.

Flipsen H (1995) Pathogenesis induced by (recombinant) baculoviruses in insects. Ph.D. thesis. Agricultural University in Wageningen, The Netherlands.

Fraser MJ (1986a) Transposon-mediated mutagenesis of baculoviruses: transposon shuttling and implications for speciation. Ann. Entomol. Soc. Amer. 79(5): 773–783.

Fraser MJ (1986b) Ultrastructural observations of virion maturation in *Autographa californica* nuclear polyhedrosis virus infected *Spodoptera frugiperda* cell cultures. J Ultrastruct Mol Struct Res 95(1–3): 189–195.

Fraser MJ & Gary L *et al.* (1995) Assay for movement of lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. Virology 211: 397–407.

Fraser MJ & Smith GE *et al.* (1983) Aquisition of host cell DNA sequences by Baculoviruses; Relationships between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis virus. J. Virol. 47: 287–300.

Friesen PD (1993) Invertebrate transposable elements in the baculovirus genome: characterization and significance. In: "Parasites and Pathogens of Insects" Vol. 2, 147–178. Academic Press.

Friesen PD & Miller LK (1986) The regulation of baculovirus gene expression. Curr. Topics in Microbiol. Immunol. 131: 31–49.

Friesen PD & Nissen MS (1990) Gene organization and transcription of TED a lepidopteran retrotransposon integrated within the baculovirus genome. Mol Cell Biol 10(6): 3067–3077.

Friesen PD & Rice WC *et al.* (1986) Bidirectional transcription from a solo long terminal repeat of the retrotransposon TED: Symmetrical RNA start sites. Mol. Cell. Biol. 6(5): 1599–1607.

Fuchs LY & Woods MS *et al.* (1983) Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: A novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. J. Virol. 48(3): 641–646.

Funk CJ & Consigli RA (1993) Phosphate cycling on the Basic Protein of *Plodia interpunctella* Granulosis Virus. Virology 193(1): 396–402.

Giacca M & Gutierrez MI *et al.* (1992) A human binding site for transcription factor USF-MLTF mimics the negative regulatory element of Human Immunodeficiency Virus type 1. Virology 186(1): 133–147.

Gijzen M & Roelvink P *et al.* (1995) Characterization of viral enhancing activity from Trichoplusia ni granulosis virus. J Invertebr Pathol 65(3): 289–294.

Glocker B & Hoopes RR *et al.* (1993) In vitro transcription from baculovirus late gene promoters: Accurate mRNA initiation by nuclear extracts prepared from infected *Spodoptera frugiperda* cells. J Virol 67(7): 3771–3776.

Gombart AF & Pearson MN *et al.* (1989) A baculovirus polyhedral envelope-associated protein: genetic location, nucleotide sequence, and immunocytochemical characterization. Virology 169(1): 182–193.

Granados RR (1978) Early events in the infection of *Heliothis tea* midgut cells by a baculovirus. Virology 90: 170–174.

Granados RR & Federici BA (eds.) (1986) The Biology of Baculoviruses. CRC Press. Boca Raton, Florida.

Granados RR & Lawler KA (1981) In vivo pathway of *Autographa californica* baculovirus invasion and infection. Virology 108: 297–308.

Grula MA & Buller PL *et al.* (1981) Alpha amanitin-resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. J. Virol. 38: 916–921.

Guarino LA (1990) Identification of a viral gene encoding a ubiquitin-like protein. Proc Natl Acad Sci USA 87(1): 409–413.

Guarino LA & Dong W (1994) Functional dissection of the Autographa californica nuclear polyhedrosis virus enhancer element hr5. Virology 200(2): 328–335.

Guarino LA & Gonzalez MA *et al.* (1986) Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. J. Virol. 60(1): 224–229.

Guarino, LA, Smith, G *et al.* (1995) Ubiquitin is attached to membranes of baculovirus particles by a novel type of phospholipid anchor. Cell 80: 301–309.

Guarino LA & Smith M (1992) Regulation of delayed-early gene transcription by dual TATA boxes. J. Virology 66: 3733–3739.

Guarino LA & Summers MD (1986a) Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. J. Virol. 57(2): 563–571.

Guarino LA & Summers MD (1986b) Interspersed homologous DNA of Autographa californica nuclear polyhedrosis virus enhances delayed-early gene expression. J. Virol. 60(1): 215–223,

Guarino LA & Summers MD (1987) Nucleotide sequence and temporal expression of a baculovirus regulatory gene. J. Virol. 61(7): 2091–2099.

Guzo D & Rathburn H *et al.* (1992) Viral and Host Cellular Transcription in *Autographa californica* Nuclear Polyhedrosis Virus-Infected Gypsy Moth Cell Lines. J Virol 66(5): 2966–2972.

Hang X & Dong W *et al.* (1995) The *lef–3* Gene *ofAutographa calfonica* Nuclear Polyhedrosis Virus Encodes a Single-Stranded DNA-Binding Protein. J. Virology 69(6): 3924–3928.

Harrison RL & Summers MD (1995a) Biosynthesis and Localization of the *Autographa californica* Nuclear Polyhedrosis Virus 25K Gene Product. Virology 208: 279–288.

Harrison RL & Summers MD (1995b) Mutations in the Autographa californica multinucleocapsid nuclear polyhedrosis virus 25 kDa protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhanced virus production. J. Gen. Virol. 76: 1451–1459.

Hashimoto Y & Corsaro BG *et al.* (1991) Location and nucleotide sequence of the gene encoding the viral enhancing factor of the *Trichoplusia ni* granulosis virus. J Gen Virol 72(11): 2645–2652.

Hawtin RE & Arnold K *et al.* (1995) Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. Virology 212(2): 673–685.

Hay BA & Wolff T *et al.* (1994) Expression of baculovirus P35 prevents cell death in Drosophila. Development 120(8): 2121–2129.

Hershberger PA & Dickson JA *et al.* (1992) Site-specific mutagenesis of the 35-kilodalton protein gene encoded by Autographa californica nuclear polyhedrosis virus: cell line- specific effects on virus replication. J Virol 66(9): 5525–5533.

Hill JE & Kuzio J *et al.* (1992) Sequence of the v-cath gene of *Chroistoneura fumiferana* nuclear polyhedrosis virus. GenBank Acc.No M97906.

Hohmann AW & Faulkner P (1983) Monoclonal antibodies to baculovirus structural proteins: Determination of specificities by Western blot analysis. Virology 125(2): 432–444.

Hong T & Braunagel SC *et al.* (1994) Transcription, Translation, and Cellular Localization of PDV-E66: A Structural Protein of the PDV Envelope of Autographa californica Nuclear Polyhedrosis Virus. Virology 204(1): 210–222.

Hooft van Iddekinge B & Smith GE *et al.* (1983) Nucleotide sequence of the polyhedrin gene of Autographa californica nuclear polyhedrosis virus. Virology 131(2): 561–565.

Horton HM & Burand JP (1993) Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J Virol 67(4): 1860–1868.

Hultmark D & Klemenz R *et al.* (1986) Translational and transcriptional control elements in the untranslated leader of the heat shock gene hsp22. Cell 44: 429–438.

Jarvis DL & Finn EE (1995) Biochemical analysis of the N-glycosylation pathway in baculovirus-infected lepidopteran insect cells. Virology 212: 500–511.

Jarvis DL & Garcia A (1994) Biosynthesis and Processing of the *Autographa californica* Nuclear Polyhedrosis Virus gp64 Protein. Virology 205: 300–313.

Jehle JA & Fritsch E *et al.* (1995) TC14.7: A novel lepidopteran transposon found in Cydia pomonella granulosis virus. Virology 207(2): 369–379.

Kamita SG & Maeda S (1993) Inhibition of Bombyx Mori Nuclear Polyhedrosis Virus NPV Replication By the Putative DNA Helicase Gene of *Autographa californica* NPV. J Virol 67(10): 6239–6245.

Kawamoto F& Kumada N *et al.* (1977) Envelopment of the Nuclear Polyhedrosis Virus of the oriental tussock moth, *Euproctis subflava.* Virology 77: 867–871.

King LA & Possee RD (1992) The Baculovirus Expression System a Laboratory Guide. King LA & RD Possee, The Baculovirus Expression System: a Laboratory Guide. Xiv+229p. Chapman and Hall: London, England, Uk; New York, New York, Usa. lllus. ISBN 0 412 37150 2. 0, XIV+229P.

Kogan PH & Blissard GW (1994) A baculovirus *gp64* early promoter is activated by host transcription factor binding to CACGTG and GATA elements. J. Virology 68(2): 813–822.

Kogan PH & Chen X *et al.* (1995) Overlapping TATA-Dependent and TATA-Independent Early Promoter Activities in the Baculovirus *gp64 envelope fusion protein* Gene. J. Virology 69: 1452–1461.

Kool M & Van Den Berg PMMM *et al.* (1993a) Location of two putative origins of DNA replication of *Autographa californica* Nuclear Polyhedrosis Virus. Virology 192: 94–101.

Kool M & Vlak JM (1993) The structural and functional organization of *the Autographa californica* nuclear polyhedrosis virus genome. Archives of Virology 130: 1–16.

Kool M & Voeten JTM *et al.* (1993b) Identification of seven putative origins *of Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus DNA replication. Journal of General Virology 74(12): 2661–2668.

Kool M & Voeten JTM *et al.* (1994) Functional mapping of regions of the *Autographa californica* nuclear polyhedrosis viral genome required for DNA replication. Virology 198(2): 680–689.

Kovacs GR & Choi J *et al.* (1992) Functional dissection of the *Autographa californica* nuclear polyhedrosis virus immediate-early 1 transcriptional regulatory protein. J Virol 66: 7429–7437.

Kovacs GR & Guarino LA *et al.* (199la) Identification of spliced baculovirus RNAs expressed late in infection. Virology 185(2): 633–643.

Kovacs GR & Guarino LA *et al.* (1991b) Novel regulatory properties of the IE1 and IEO transactivators encoded by the baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus. J Virol 65(10): 5281–5288.

Krappa R *&* Behn KA *et al.* (1992) Differential factor binding at the promoter of early baculovirus gene *pe38* during viral infection: GATA motif is recognized by an insect protein. J Virol 66(6): 3494–3503.

Krappa R & Knebel MD (1991) Identification of the very early transcribed baculovirus gene PE–38. J Virol 65(2): 805–812.

Kuzio J &Jaques R *et al.* (1989) Identification of p74 a gene essential for virulence of baculovirus occlusion bodies. Virology 173(2): 759–763.

Kuzio J & Rohel DZ *et al.* (1984) Nucleotide sequence of the p 10 polypeptide gene of Autographa californica nuclear polyhedrosis virus. Virology 139(2): 414–418.

Leisy DJ & Rasmussen C *et al.* (1995) The *Autographa californica* Nuclear Polyhedrosis Virus Homologous Region la: Identical Sequences Are Essential for DNA Replication Activity and Transcriptional Enhancer Function. Virology 208: 742–752.

Leisy DJ & Rohrmann GF (1993) Characterization of the replication of plasmids containing *hr* sequences in baculovirus-infected *Spodopterafrugiperda* cells. Virology 196(2): 722–730.

Leisy DJ & Rohrmann GF *et al.* (1986) Nucleotide sequencing and transcriptional mapping of the Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus p10 gene. Virology 153(2): 157–167.

Lerch RA & Friesen PD (1992) The baculovirus-integrated retrotransposon TED encodes gag and pol proteins that assemble into viruslike particles with reverse transcriptase. J Virol 66(3): 1590–1601.

Li Y & Miller LK (1995) Expression and functional analysis of a baculovirus gene encoding a truncated protein kinase homolog. Virology 206: 314–323.

Liu JC & Maruniak JE (1995) Nucleotide sequence and transcriptional analysis of the gp41 gene of Spodoptera frugiperda nuclear polyhedrosis virus. J Gen Virol 76(Part 6): 1443–1450.

Lu A & Carstens EB (1991) Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis virus. Virology 181(1): 336–347.

Lu A & Carstens EB (1993) Immediate-early baculovirus genes transactivate *the pi43* gene promoter *of Autographa californica* nuclear polyhedrosis virus. Virology 195(2): 710–718.

Lu A & Miller LK (1995) The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. J. Virology 69: 975–982.

Ma SW & Corsaro BG *et al.* (1993) Cloning and sequence analysis of a P40 structural protein gene of *Helicoverpa zea* Nuclear Polyhedrosis Virus. Virology 192(1): 224–233.

Maeda S & Kamita SG *et al.* (1993) Host Range Expansion of *Autographa californica* Nuclear Polyhedrosis Virus NPV Following Recombination of a 0.6-Kilobase-pair DNA Fragment Originating from *Bombyx Mori* NPV. J Virol 67(10): 6234–6238.

Majima K & Kobara R *et al.* (1993) Divergence and Evolution of Homologous Regions of Bombyx-mori Nuclear Polyhedrosis Virus. J Virol 67(12): 7513–7521.

McLachlin JR & Miller LK (1994) Identification and characterization of *vlf–1,* a baculovirus gene involved in very late gene expression. J. Virology 68: 7746–7756.

Miller LK (1995) Insect viruses. *In* "Virology" (BN Fields, DM Knipe & P Howley, Eds.), pp. 533–556. Lippincott-Raven, New York.

Miller DW & Miller LK (1982) A virus mutant with an insertion of a copia-like transposable element. Nature (London) 299: 562–564.

Miller LK & Jewell JE *et al. (*1981) Baculovirus Induction of a DNA Polymerase. J. Virol. 40(1): 305–308.

Monsma SA & Blissard GW (1995) Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 Envelope Fusion Protein. J. Virol. 69(4): 2583–2595.

Monsma SA & Oomens AGP *et al.* (1996) The GP64 Envelope Fusion Protein is an essential baculovirus protein required for cell to cell transmission of infection. J. Virology 70: 4608–4616.

Morris TD & Miller LK (1994) Mutational analysis of a baculovirus major late promoter. Gene 140(2): 147–153.

Morse MA & Marriott AC *et al. (*1992) The glycoprotein of Thogoto Virus (a tick-borne orthomyxo-like virus) is related to the baculovirus glycoprotein gp64. Virology 186: 640–646.

O'Reilly DR (1995) Baculovirus-encoded ecdysteroid UDP-glucosyltransferases. Insect Biochem. Molec. Biol. 25: 541–550.

O'Reilly DR & Brown MR *et al.* (1992a) Alteration of ecdysteroid metabolism due to baculovirus infection of the fall armyworm *Spodoptera frugiperda* host ecdysteroids are conjugated with galactose. Insect Biochem Mol Biol 22(4): 313-320.

O'Reilly DR & Miller LK (1989) A baculovirus blocks insect molting by producing ecdysteroid UDPglucosyltransferase. Science 245: 1110–1112.

O'Reilly DR & Miller LK (1990) Regulation of Expression of a Baculovirus Ecdysteroid UDPglucosyltransferase Gene. J. Virol 64; 1321–1328.

O'Reilly DR & Miller LK *et al.* (1992b) "Baculovirus expression vectors, a laboratory manual." WH Freeman and Co., New York.

Ohkaa T & Majima K *et al.* (1994) A cysteine protease encoded by the baculovirus Bombyx mori nuclear polyhedrosis virus. Journal of Virology. 68(10): 6619–6625.

Ooi B & Miller LK (1988) Regulation of host RNA levels during baculovirus infection. Virology 166(2): 515–523.

Ooi BG & Rankin C *et al.* (1989) Downstream sequences augment transcription from the essential initiation site of a baculovirus polyhedrin gene. J. Mol. Biol. 210: 721–736.

Oomens AGP & Monsma SA *et al.* (1995) The baculovirus GP64 Envelope Fusion Protein: Synthesis, Oligomerization, and Processing. Virology 209: 592–603.

Orkin SH (1992) GATA-binding transcription factors in hematopoietic cells. Blood 80(3): 575–581.

Passarelli AL & Miller LK (1993) Identification of genes encoding late expression factors located between 56.0 and 65.4 map units of the Autographa californica nuclear polyhedrosis virus genome. Virology 197(2): 704–714.

Pearson M & Bjornson R *et al.* (1992) The *Autographa californica* baculovirus genome: evidence for multiple replication origins. Science (Washington D C) 257(5075): 1382–1384.

Pham DQD & Sivasubramanian N (1992) In-vivo transcriptional analysis of three baculovirus genes: Evidence of homology between viral and host transcripts. Virology 190(1): 288–297.

Portela A & Jones LD *et al.* (1992) Identification of viral structural polypeptides of Thogoto virus, a tick-borne orthomyxo-like virus and functions associated with the glycoprotein. J Gen Virol 73(11): 2823–2830.

Possee RD & Sun TP *et al.* (1991) Nucleotide Sequence of the *Autographa californica* Nuclear Polyhedrosis 9.4 Kbp EcoRI-I and R Polyhedrin Gene Region. Virology 185(1): 229–241.

Pullen SS & Friesen PD (1995a) The CAGT Motif Functions as an Initiator Element during Early Transcription of the Baculovirus Transregulator *ie–1.* J. Virology 69: 3575–3583.

Pullen SS & Friesen PD (1995b) Early transcription of the *ie–1* transregulator gene of *Autographa californica* nuclear polyhedrosis virus is regulated by DNA sequences within its 5′ noncoding leader region. J Virol 69(1): 156–165.

Purnell BA & Emanuel PA *et al.* (1994) TFIID sequence recognition of the initiator and sequences farther downstream in *Drosophila* class II genes. Genes & Development 8(7): 830–842.

Rabizadeh S & Lacount DJ *et al.* (1993) Expression of the baculovirus p35 gene inhibits mammalian neural cell death. Journal of Neurochemistry 61(6): 2318–2321.

Rankin C & Ooi BG *et al.* (1988) Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression. Gene 70(1): 39–49.

Reach M & Xu LX *et al.* (1991) Transcription from the Adenovirus Major Late Promoter uses redundant activating elements. EMBO J. 10(11): 3439–3446.

Reilly LM & Guarino LA (1994) The *pk–1* gene *of Autographa californica* multinucleocapsid nuclear polyhedrosis virus encodes a protein kinase. Journal of General Virology. 75(11): 2999–3006.

Rice WC & Miller LK (1986) Baculovirus transcription in the presence of inhibitors and in nonpermissive Drosophila cells. Virus Research 6: 155–172.

Roberts SR & Manning JS (1993) The major envelope glycoprotein of the extracellular virion *of Autographa californica* Nuclear Polyhedrosis Virus possesses at least three distinct neutralizing epitopes. Virus Res 28(3): 285–297.

Roberts TE (1989) Ph.D. Dissertation. Queen's University, Kingston, Ontario, Canada.

Roberts TE & Faulkner P (1989) Fatty acid acylation of the 67K envelope glycoprotein of a baculovirus *Autographa californica* nuclear polyhedrosis virus. Virology 172(1): 377–381.

Rodems SM & Friesen PD (1993) The *hr5* transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. J. Virology 67(10): 5776–5785.

Rodems SM & Friesen PD (1995) Transcriptional enhancer activity of *hr5* requires dual-palindrome half sites that mediate binding of a dimeric form of the baculovirus transregulator IE1. J. Virology 69: 5368-5375.

Rohrmann GF (1986) Polyhedrin structure. J. Gen. Virol. 67: 1499–1513.

Rohrmann GF (1992) Baculovirus structural proteins. J. Gen. Virol. 73:749–761.

Roy N & Mahadevan MS *et al.* (1995) The Gene for Neuronal Apoptosis Inhibitory Protein Is Partially Deleted in Individuals with Spinal Muscular Atrophy. Cell 80: 167–178.

Russell RLQ & Rohrmann GF (1990) The *p6.5* gene region of a Nuclear Polyhedrosis Virus of *Orgyia pseudotsugata:* DNA sequence and transcriptional analysis of four late genes. J Gen Virol 71(3): 551–560.

Russell RLQ & Rohrmann GF (1993a) A 25-kda protein is associated with the envelopes of occluded baculovirus virions. Virology 195(2): 532–540.

Russell RLQ & Rohrmann GF (1993b) Nucleotide sequence of the ubiquitin–39k gene region from the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. J Gen Virol 74(6): 1191–1195.

Slack JM & Kuzio J *et al.* (1995) Characterization of v-cath, a cathepsin L-like proteinase expressed by the baculovirus AcNPV. J. Gen. Virology 76: 1091–1098.

Sugimoto A & Friesen PD *et al.* (1994) Baculovirus p35 prevents developmentally programmed cell death and rescues a ced–9 mutant in the nematode Caenorhabditis elegans. EMBO J. 13(9): 2023–2028.

Summers MD (1971) Electron Microscopic Observations of Granulosis Virus Entry, Uncoating and Replication Processes during Infection of the Midgut Cells of *Trichoplusia ni.* J. Ultrastr. Research 35: 606–625.

Teakle RE (1969) A nuclear-polyhedrosis virus of *Anthela varia* (Lepidoptera: Anthelidae). J. Invertebr. Pathol. 14: 18–27.

Theilmann DA & Chantler JK *et al.* (1996) Characterization of a highly conserved baculovirus structural protein that is specific for occlusion-derived virions. Virology 218: 148–158.

Theilmann DA & Stewart S (1991) Identification and characterization of the IE–1 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology 180(2): 492–508.

Theilmann DA & Stewart S (1992) Molecular analysis of the trans-activating *ie–2* gene of *Orgyia pseudotsugata* Multicapsid Nuclear Polyhedrosis Virus. Virology 187(1): 84–96.

Theilmann DA & Stewart S (1993) Analysis of the *Orgyia pseudotsugata* multicapsid Nuclear Polyhedrosis Virus trans-activators IE-1 and IE-2 using monoclonal antibodies. J Gen Virol 74(9): 1819–1826.

Todd JW & Passarelli AL *et al.* (1995) Eighteen baculovirus genes, including lef–11, p. 35, 39K, and p47, support late gene expression. J. Virology 69: 968–974.

Tomalski MD & Eldridge R *et al.* (1991) A baculovirus homolog of a copper zinc superoxide dismutase gene. Virology 184(1): 149–161.

Tweeten KA & Bulla LA *et al.* (1980a) Characterization of an extremely basic protein derived from granulosis virus nucleocapsid. J. Virol. 33: 866–876.

Tweeten KA & Bulla LA *et al.* (1980b) Structural polypeptides of the granulosis virus of *Plodia interpunctella.* J. Virol. 33: 877–886.

Van-Oers MM & Flipsen JTM *et al.* (1993) Functional domains of the p10 protein of *Autographa califarnica* nuclear polyhedrosis virus. J Gen Virol 74(4): 563–574.

Van-Oers MM & Flipsen JTM *et al.* (1994) Specificity of baculovirus p10 functions. Virology 200(2): 513–523.

Vaux DL & Haecker G *et al.* (1994) An evolutionary perspective on apoptosis. Cell. 76(5): 777–779.

Vialard JE & Richardson CD (1993) The 1629-nucleotide open reading frame located downstream of the *Autographa californica* Nuclear Polyhedrosis Virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. J Virol 67(10): 5859–5866.

Vogel SS & Leikina EA *et al.* (1993) Lysophosphatidylcholine reversibly arrests exocytosis and viral fusion at a stage between triggering and membrane merger. Journal of Biological Chemistry. 268(34): 25764–25768.

Volkman LE (1986) The 64K envelope protein of budded *Autographa californica* nuclear polyhedrosis virus. Curr. Top. Microbiol. Immunol. 131: 103–118.

Volkman LE & Blissard GW *et al.* (1995) Baculoviridae: Taxonomic structure and properties of the family. *In* Classification and nomenclature of viruses: Sixth report of the international committee for the taxonomy of viruses. Archives of Virology Supplement 10: 104–113.

Volkman LE & Goldsmith PA (1984) Budded *Autographa californica* NPV 64K protein: further biochemical analysis and effects of postimmunoprecipitation sample preparation conditions. Virology 139: 295–302.

Volkman LE & Goldsmith PA (1985) Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: Inhibition of entry by adsorptive endocytosis. Virology 143(1): 185–195.

Volkman LE & Goldsmith PA *et al.* (1987) Evidence for microfilament involvement in budded Autographa californica nuclear polyhedrosis virus production. Virology 156(1): 32–39.

Volkman LE & Goldsmith PA *et al.* (1984) Neutralization of budded *Autographa californica* NPV by a monoclonal antibody: Identification of the target antigen. Virology 133(2): 354–362.

Volkman LE & Summers MD (1977) *Autographa californica* nuclear polyhedrosis virus: Comparitive infectivity of the occluded, alkali-liberated, and nonoccluded forms, J, Invertebr. Pathol. 30: 102–103.

Wang HGH & Fraser MJ *et al.* (1989) Transposon mutagenesis of baculoviruses analysis of TFP3 lepidopteran transposon insertions at the fp locus of nuclear polyhedrosis viruses. Gene (Amst) 81(1): 97–108.

Wang P & Hammer DA *et al.* (1994) Interaction of Trichoplusia ni granulosis virus-encoded enhancin with the midgut epithelium and peritrophic membrane of four lepidopteran insects. Journal of General Virology 75(8): 1961–1967.

White JM (1992) Membrane fusion. Science 258(5084): 917–924.

Whitford M & Faulkner P (1992a) Nucleotide sequence and transcriptional analysis of a gene encoding gp41 a structural glycoprotein of the baculovirus *Autographa califarnica* nuclear polyhedrosis virus. J Virol 66(8): 4763–4768.

Whitford M & Faulkner P (1992b) A structural polypeptide of the baculovirus *Autographa californica* nuclear polyhedrosis virus contains O-linked N-acetylglucosamine. J Virol 66(6): 3324–3329.

Whitford M & Stewart S *et al.* (1989) Identification and sequence analysis of a gene encoding *gp67,* an abundant envelope glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J Virol 63(3): 1393–1399.

Whitt MA & Manning JS (1988) A phosphorylated 34-kDa protein and a subpopulation of polyhedrin are thiol linked to the carbohydrate layer surrounding a baculovirus occlusion body. Virology 163(1): 33–42.

Wickham T & Granados RR *et al.* (1990) General analysis of receptor-mediated viral attachment to cell surfaces. Biophys. J. 58: 1501–1516.

Williams GV & Rohel DZ *et al.* (1989) A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion-deletion mutants. J Gen Virol 70(1): 187–202.

Wilson ME & Consigli RA (1985a) Characterization of a protein kinase activity associated with purified capsids of the granulosis virus infection *Plodia interpunctella.* Virology 143: 516–525.

Wilson ME & Consigli RA (1985b) Functions of a protein kinase activity associated with purified capsids of the granulosis virus infecting *Plodia interpunctella.* Virology 143: 526–535.

Wilson ME & Mainprize TH *et al.* (1987) Location, transcription, and sequence of a baculovirus gene encoding a small arginine-rich polypeptide. J. Virol. 61(3): 661–666.

Winstanley D & Crook NE (1993) Replication Of Cydia Pomonella Granulosis Virus In Cell Cultures. J Gen Virol 74(8): 1599–1609.

Wood HA (1980) *Autographa californica* nuclear polyhedrosis virus-induced proteins in tissue culture. Virology 102: 21–27.

Wu X & Stewart S *et al.* (1993) Alternative transcriptional initiation as a novel mechanism for regulating expression of a baculovirus trans activator. J Virol 67(10): 5833–5842.

Xu B & Yoo S *et al.* (1995) Differential Transcription of Baculovirus Late and Very Late Promoters: Fractionation of Nuclear Extracts by Phosphocellulose Chromatography. J. Virology 69: 2912–2917.

Yang CL & Stetler DA *et al.* (1991) Structural comparison of the *Autographa californica* nuclear polyhedrosis virus-induced RNA polymerase and the three nuclear RNA polymerases from the host *Spodoptera frugiperda.* Virus Res 20(3): 251–264.

Yoo S & Guarino LA (1994) The Autographa californica nuclear polyhedrosis virus ie2 gene encodes a transcriptional regulator. Virology 202(2): 746–753.

Young JC & Mackinnon EA *et al.* (1993) The architecture of the virogenic stroma in isolated nuclei of *Spodoptera frugiperda* cells in vitro infected by *Autographa californica* nuclear polyhedrosis virus. J Struct Biol 110(2): 141–153.

Zanotto PM & Kessing BD *et al.* (1993) Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. J Invertebr Pathol 62(2): 147–164.

Zimmerberg J & Vogel SS *et al.* (1993) Mechanisms of membrane fusion. Ann Rev Biophys Biomol Structure 22: 433–466.

Zuidema D & Van Oers MM *et al.* (1993) Nucleotide sequence and transcriptional analysis of the p10 gene of *Spodoptera exigua* nuclear polyhedrosis virus. J Gen Virol 74: 1017–1024.

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Replication patterns and cytopathology of cells infected with baculoviruses

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Introduction

Baculoviruses are a large family of DNA containing viruses which exclusively infect arthropod hosts, and comprise the nuclear polyhedrosis (NPV), granulosis (GV), and non-occluded (NOB) viruses (Francki *et al.*, 1991). A factor that contributes to complexity in discussion of their replication pattern is that the NPV and GV type baculoviruses have a biphasic replication cycle (Figure 1) and exist as two phenotypic forms which carry identical genetic information within their nucleocapsids. These phenotypes play specific roles in natural (horizontal) transmission between insects and systemic spread within an individual insect larva (Faulkner, 1981; Bilimoria, 1991). Baculoviruses are potentially useful agents for the control of important insect pests in ecologically-based pest management field programmes where biological agents are used alone or with pesticides and other chemical agents. In addition recombinant baculoviruses are also used to express diverse eucaryotic genes in insect cells for use in research and an increasing number of biotechnological applications. (Miller, 1989; Luckow, 1991;O'Reilly *et al.*, 1992). This chapter will focus on the cellular biology of baculovirus replication, in particular nuclear polyhedrosis viruses, and will only deal in a peripheral manner with the molecular aspects of gene expression and regulation. The time scale of morphogenic events is related to the type species, *Autographa californica* MNPV, unless otherwise noted.

An overview of baculovirus replication

In nature, NPV infection begins when an insect feeds on material contaminated with baculovirus occlusion **b**odies (**OB**). These structures are formed in nuclei of infected cells and are sometines referred to as **polyhedra** due to their crystalline shape and appearance in the light microscope. Approximately 95% of the mass of OB is made up of a crystalline lattice of the protein **polyhedrin;** embedded within the lattice lie bundles of virions. The occlusion bodies are resistant to putrefaction and disruption by a variety of chemical agents (Benz, 1986), or physical treatments such as freezing and dessication (Jacques, 1985), but are dissolved in the alkali conditions encountered in the insect midgut (pH 9.5–11.5) (Granados & Williams, 1986), and release **p**olyhhedron **d**erived **v**irus particles (**PDV**) which may then attatch to and subsequently infect the midgut columnar epithelial cells. At least two OB-associated factors have been implicated as having supporting roles in the dissolution and infection process. Alkaline proteases, associated with insect derived OB, are probably endogenous enzymes of the insect host gut (Rubenstein & Polson, 1983; Nagata & Tanada, 1983). In addition, a proteinaceous factor associated with OB (Derksen & Granados, 1988) may aid viral penetration of the insect peritrophic membrane, which is a chitin rich selective barrier that lines the midgut.

PDV enter the epithelial cells by fusion of their envelope with the plasma membrane at brush border microvilli (Kawanishi *et al.*, 1972; Granados, 1978; Granados & Williams, 1986). Neutralization of infection by anti-PDV serum has been reported (Keddie & Volkman, 1985), implying that the process of PDV attachment and entry may be receptor mediated. After membrane fusion, virus nucleocapsids transverse the cytoplasm to the nuclear membrane where they probably gain entry to the nucleus through nuclear pores (Granados & Lawler, 1981; Federici, 1986). Subse-

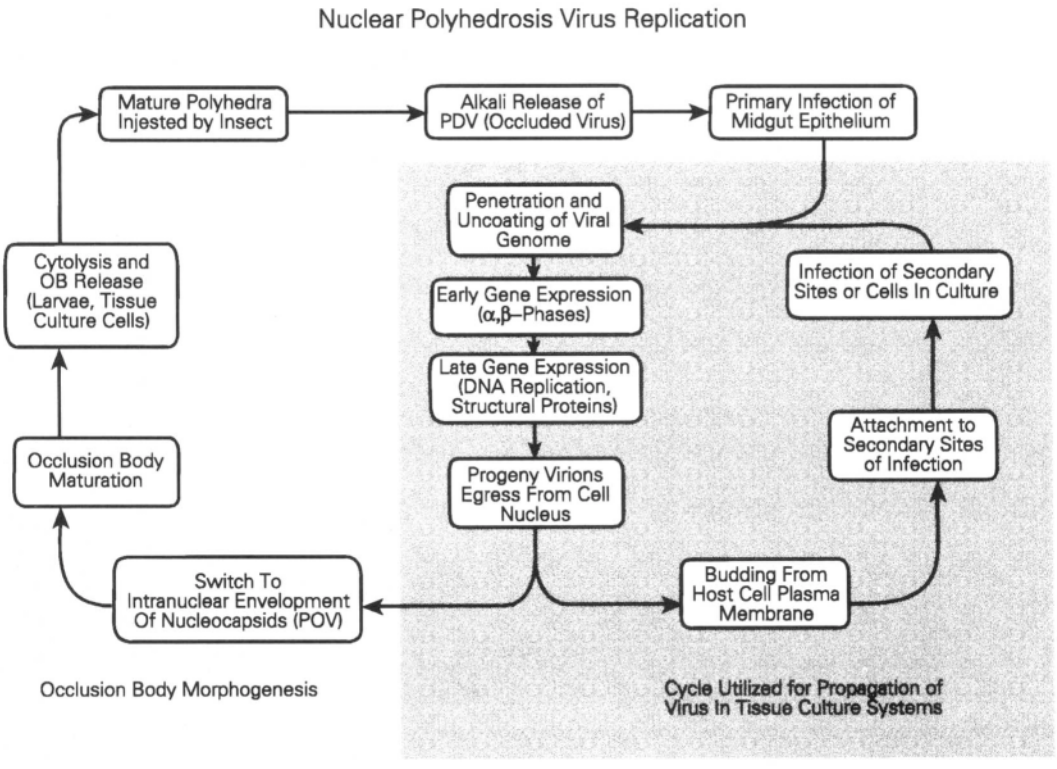

Figure 1. Nuclear polyhedrosis virus replication.

quently, viral nucleocapsids uncoat into the nucleoplasm and the genomic DNA is exposed to the host cell transcription machinery. No viral proteins associated with the nucleocapsid are required for the initiation of transcription (mRNA production) or translation (protein synthesis) of a group of early viral genes since these are recognised by cell components (Carstens *et al.*, 1980; Kelly & Wang, 1981).

Ultrastructural and histochemical studies in cell culture have revealed that replication of viral DNA is associated with the appearance of a dense virogenic stroma within the nucleus and that progeny virus nucleocapsids are assembled within this structure (Federici, 1986; Granados & Williams, 1986). Initially the viral DNA may be associated with host nucleosomal proteins; however, as the infection progresses the host proteins are displaced by a virus-encoded DNA-binding protein (Wilson & Miller, 1986). Nucleocapsid assembly occurs by condensation of the virus genome to a tight helicoid nucleoprotein complex in a process thought to be mediated by the DNA binding protein. Hollow capsid shells have been seen aligned end-on to the virogenic stroma and a subsequent maturation step results in the filling of the capsids with the nucleoprotein complex to produce nucleocapsids (Bassemir *et al.*, 1983; Fraser, 1986).

In cell culture, by 24 hours post infection, copious amounts of newly formed nucleocapsids are seen leaving the nucleus in the first phase of virus progeny development. The process involves budding through the nuclear membrane (synhymenosis) (Kawamoto *et al.*, 1977), subsequent loss of the nuclear membrane-derived envelope during traversal of the cytoplasm, and final extrusion of nucleocapsids by budding through the host cell plasma membrane at regions modified by incorporation of virus encoded proteins (Volkman, 1986). At this stage, the infectious particles are known as budded virus (BV). BV released from the basolateral surface of gut epithelial cells effect systemic spread of infection *in vivo* (Keddie *et al.*, 1989).

The process of viral entry has been subjected to greater study for BV than for PDV and appears to be mechanistically different (Volkman & Keddie, 1990). BV are the form of the virus responsible for spread

of the infection within the insect and they have broad target organ specificity (Keddie *et al.*, 1989); this phenotypic form is also the agent used in propagation of the virus in cell culture applications (Refer to Figure 1). The principle mode of entry is by receptor mediated adsorptive endocytosis (Volkman & Goldsmith, 1985). Once the virus is adsorbed onto the cell surface, it is internalized within clathrin coated vesicles (Wickham *et al.*, 1992). After uncoating of the vesicle (endosome), nucleocapsids gain entry into the host cell cytoplasm by fusion of the virus envelope with the endosome membrane (Volkman, 1986). Fusion of primary lysosomes to virus-containing endosomes and concomitant pH changes probably triggers the membrane fusion process. BV entry into cells by direct fusion at the plasma membrane, in the manner of PDV, may occur, albeit inefficiently. After this step, the nucleocapsids may proceed with the infectious process as will be described later.

At later stages in the infection cycle, BV synthesis is curtailed in both the insect and in tissue culture, in favour of synthesis of the PDV phenotype. Large numbers of nucleocapsids accumulate in the intranuclear ring zone (preistromal compartment) and become enveloped, either individually or in bundles. The origin of the PDV envelope is somewhat uncertain. Components may be synthesized *de novo* (Stoltz *et al.*, 1973; Mackinnon *et al.*, 1974); however, the source material is most likely derived from the host cell inner nuclear membrane and subsequently modified (Tanada & Hess, 1976; Summers & Arnott, 1969).

Also, late in the infection cycle, polyhedrin synthesis accelerates and the protein is transported into the nucleus where it begins to crystallize. Concomitantly a second protein, p10 is also produced in abundance and condenses as distinct fibrillar structures in both the cytoplasm and nucleus (Williams *et al.*, 1989). During the process of polyhedrin condensation PDV become embedded within the matrix. Maturation of occlusion bodies is completed by their envelopment in a polyhedral calyx composed primarily of sugars and at least one phosphoprotein (Minion *et al.*, 1979; Whitt & Manning, 1988). Eventual insect death due to polyhedrosis disease results in release of OB and contamination of the surrounding substrate. Release of OB is probably accelerated due to the action of a virus-encoded cysteine proteinase (Slack *et al.*, 1995) and a chitinase (R.D. Possee *pers. comm.*) which may cause dissolution of the larval tissues and cuticle, respectively. After death and lysis of the host insect tissues and cuticle, OB protect PDV prior to ingestion by other larvae. Occlusion bodies play an important role in virus persistence in populations of insects that have seasonal feeding cycles (Jaques, 1977; Jaques, 1985).

Baculovirus phenotypes

NPV have two genotypically identical phenotypic forms, each with a specific function in the spread of infection; from host to host by the **PDV**, and systemically between tissues or cells within an insect as the **BV**. The difference between the phenotypes resides exclusively in the envelope and tegument regions of the virion, and the nucleocapsid core is common to both phenotypes (Rohrmann, 1992). Viral envelopes can be removed using nonionic detergents without damaging nucleocapsid structure or composition (Wilson & Consigli, 1985a; Braunagel & Summers, 1994; Dobos & Cochran, 1980; Stiles *et al.*, 1983; Kelly & Lescott, 1983).

Immunochemistry-based techniques have been used to demonstrate that each form of virion has distinct protein species (Volkman, 1983) and marked differences in infectivity *in vivo* and *in vitro* (Keddie & Volkman, 1985; Hink, 1982). BV gains entry to host cells by adsorptive endocytosis (Volkman & Goldsmith, 1985; Volkman *et al.*, 1986). This form possesses an envelope glycoprotein, gp67 (Whitford *et al.*, 1989) which is important in infectivity: a monoclonal antibody AcV1 can neutralize BV *in vitro* and *in vivo* by binding gp67 (Hohmann & Faulkner, 1983; Volkman *et al.*, 1984; Keddie & Volkman, 1985). Spike-like features at the apical ends of BV called peplomers are believed to be composed of multimeric gp67 (Volkman & Knudson, 1986; Volkman, 1986). The gp67 protein has a characteristic N-terminal signal sequence and a C-terminal anchoring region that are common to transmembrane proteins (Whitford *et al.*, 1989; Blissard & Rohrmann, 1989), and the molecule may be further stabilized in the membrane by covalently linked fatty acids (Roberts & Faulkner, 1989).

By contrast, PDV enters insect epithelial cells by fusion at the cell surface (Granados & Lawler, 1981). Although spikes or prominent surface glycoproteins, and molecules that could participate in adsorption, fusion, and penetration have not been identified specifically (see Rohrmann, 1992), a 25 kda peptide (Russell & Rohrmann, 1993) and two other species, PDV-E66 and PDV-E43 (Braunagel & Summers, 1994; Hong *et al.*, 1994) were identified recently as constituents of the PDV envelope. A major glycoprotein of PDV (gp41) was detected in the tegument region of the virion (Whit-

ford & Faulkner, 1992a,b) and although probably not involved in virus attachment or fusion, it may play a role in capsid entry or transport once fusion occurs. A minor peptide, p74, is associated with envelopes of PDV and was not detected in purified BV preparations (Faulkner *et al*., 1995). AcMNPV mutants lacking p74 gene generate OB that are non-infectious when fed to insects (Kuzio *et al*., 1989), although OB from the mutants are indistinguishable morphologically from those isolated from insects or cell cultures infected with the wt virus (Faulkner *et al*., 1995). Analysis of feeding experiments and ultrastructural studies point to the p74 peptide as having an essential role in initiating infection most likely at the surface of insect midgut epithelia (Faulkner *et al.,* 1995).

Baculovirus nucleocapsids

Baculovirus nucleocapsids comprise a nucleoprotein core and a protein shell (the capsid). The core contains the supercoiled viral genome complexed with a highly basic 6.9 kda protein that has been characterized in several NPV's (Wilson *et al*., 1987; Russell & Rohrmann, 1991; Maeda *et al*., 1991) and which functions to compact the large viral genome into a tight helicoid structure for efficient packaging. The 6.9k gene products are small highly basic peptides rich in arginine residues (pI 12.8–12.9) that share considerable sequence identity, and are notably serine-threonine rich. Phosphorylation of serine-threonine residues, by a nucleocapsid associated protein kinase, may be needed in the virus uncoating process (Miller *et al*., 1983; Wilson & Consigli, 1985a,b). Tyrosine residues are conserved and spaced throughout the length of the peptide. It has been proposed that the viral genome is condensed by ionic interactions between the arginine residues and the phosphopentose backbone of the DNA, while the tyrosine residues may intercalate among the stacked base pairs (Kelly *et al*., 1983).

The nucleocapsid is observed as a tubular capsid shell with distinct cap structures on each end, and enclosing an inner nucleoprotein core. These major components can be separated using dilute alkali or detergent with high salt (Wilson & Consigli, 1985a). The capsid shell is comprised of at least 9 proteins (Kelley, 1985), although the predominant species is a 39 kda protein which has been characterized for several NPV's (Thiem & Miller, 1989; Russel *et al*., 1991; Pearson *et al*., 1988; Blissard *et al*., 1989; Bjornson & Rohrmann, 1992). Western blots of PDV and BV phenotypes (Pearson *et al*., 1988) and immunocytochemistry (Russell *et al*., 1991) demonstrated p39 to be a major structural component of the capsid. Recently a 24 kda protein was also found to be evenly distributed through the nucleocapsid in both the PDV and BV phenotypes (Wolgamot *et al*., 1993), although its function is not known. In the E-strain of AcMNPV a transposable element interrupts the p24 gene without deleterious effect (Schetter *et al*., 1990), so this protein does not appear to be essential for growth in cell culture. Another capsid-associated protein (87 kda) of unknown function was found in *Orgyia pseudotsugata* MNPV (Muller *et al.,* 1990), and its 80 kda homologue in AcMNPV has also been described (Lu & Carstens, 1992). An attempt to localize p87 within the capsid using immunoelectron microscopy was not successful (Rohrmann, 1992).

End structures found on the nucleocapsids are distinct, with a flat or rounded disc on one end called a base plate (basal end), and a nipple shaped structure on the other (apical) end (Kawamoto *et al*., 1977; Federici, 1986; Volkman & Keddie, 1990), hence the nucleocapsids display polarity. The structures may reflect the mechanism of assembly of nucleocapsids, and it has been speculated that the apical end cap of the virus may mediate packing of the nucleoprotein into the capsid (Fraser, 1986). There is significant morphological evidence that the apical end may interact with membranes to trigger envelopment of nucleocapsid particles, as capsids are frequently seen aligned with membranes at the nipple end during virion morphogenesis or egress through the nuclear membrane (Kawamoto *et al*., 1977; Fraser, 1986). Electron micrographs have shown an association of the virus nucleocapsid apical end caps and the nuclear pores (Granados & Lawler, 1981).

Vialard & Richardson (1993) localized an essential 78 kda phosphoprotein to the ends of the capsid shell of AcMNPV thereby identifying the first capsid structural protein with a polarized distribution and perhaps a component of one or both of the specialized end structures. The 78 kda protein was immunolocalized to the periphery of the virogenic stroma and was present in purified nucleocapsids of PDV and BV phenotypes as both 83 kda phosphorylated and 78 kda unphosphorylated species. Partial polypeptides of 49 and 51 kda were also present later in infection, due to either alternate transcription or proteolytic processing (Vialard & Richardson, 1993), and may be present exclusively in nucleocapsids of the PDV phenotype. Attempts to delete the gene have not been successful (Possee *et al*., 1991) and in the context of the localization data available such recombinants may simply fail to produce

infectious virions. Another capsid associated protein, p87, has been identified as a component of both BV and PDV but it is not known if this protein lies within the cylindrical portion of the capsid or in an end cap (Muller *et al*., 1990).

Early cytopathology of NPV replication

Virus entry and early events

Early morphological changes to the host cell cytoskeleton have been associated with NPV entry into insect cells, and include transient alterations to microfilaments causing the formation of F-actin bundles (Charlton & Volkman, 1993a); virions are believed to migrate to the cell nucleus for uncoating by utilizing these tracts. Nucleocapsids entering insect midgut cells by infection with PDV have also been reported to migrate to the nucleus along cytoskeletal elements (Granados 1978), suggesting a common transfer mechanism and consistent with reports of identical nucleocapsid structures in the two phenotypes (Rohrmann, 1992). Virus capsids were seen in midgut cell nuclei within 2–4 hr pi *in vivo* (Granados, 1978), and were similarly seen in nuclei of cultured cells by 1–3 hr pi (Knudson & Harrap, 1976); a window of 1–4 hr post-innoculation is generally considered the time frame for penetration and uncoating of baculoviruses. Nucleocapsids are released into the cytoplasm proper by (1) PDV fusion to microvilli of midgut cells (Granados & Lawler, 1981; Horton & Burand, 1993) or (2) adsorptive endocytosis of BV and subsequent fusion of viral envelope with endosome membrane (Volkman & Goldsmith, 1985). There may be exceptions to these routes of entry, as some PDV may enter by viropexis (Adams *et al*., 1977), and some BV by fusion at the cell surface (Volkman *et al*., 1986). Kinetic studies suggest that both phenotypes bind to specific receptor structures on the target membrane in a saturable fashion (Horton & Burand, 1993; Wickham *et al*., 1992).

The induction of cytoskeletal changes is an interesting observation since physiological activation or stimulation of cells is known to promote actin assembly (Harris, 1987), and may indicate mitogenic factors associated with the virion. At the nuclear membrane, the action of a viral capsid-associated kinase is attributed to the release of the viral genome at or just inside a nuclear pore by phosphorylation of the highly basic nucleoprotein core protein p6.9 (Miller *et al*., 1983; Wilson & Consigli, 1985). Transfection of viral DNA into insect cells yields productive infection *in vitro* (Kelly & Wang, 1981; Carstens & Doerfler, 1980; Burand *et al*., 1980; Potter & Miller, 1980), and it is generally accepted that virion-associated proteins are not required to initiate transcription of immediate early genes or alter host replication machinery. However, in natural infections the nucleocapsid is targeted to a nuclear pore where the viral genome is released into the nucleoplasm. Accessory molecules associated with these functions may have pleotrophic actions which induce cytoskeletal changes. A good candidate would be the kinase activity associated with the capsid, because phosphorylation of cytoskeleton-associated proteins is known to cause alterations in the dynamics of and interaction between microtubules and microfilaments (Hirokawa, 1994).

Early transcription and utilization of host cell replication machinery

In the context of baculovirus molecular biology, transcription is initiated soon after uncoating of the viral genome by utilizing the host cell machinery (immediate early, alpha), but transcription of subsequent phases is dependant on viral gene products from previous phases. (reviewed by Blissard & Rohrmann, 1990; Friesen & Miller, 1986). Early transcription is sensitive to α-amanitin (Huh & Weaver, 1990a; Hoopes & Rohrmann, 1991), indicating initial utilization of host cell RNA pol II. Transcription becomes predominantly α-amanitin insensitive later in infection (Grula *et al*., 1981) and it is believed that the NPV genome encodes a novel RNA polymerase, although potential components were not identified by analysis of the complete AcMNPV sequence (Ayres *et al*., 1994), nor has the complete α-amanitin insensitive polymerase been purified successfully from infected cells (Grula *et al*., 1981; Fuchs *et al*., 1983; Huh & Weaver, 1990b; Yang *et al*., 1991). Recently RNA *pol*-like motifs were identified in the late expression factor 8 (lef–8) coding region (Passarelli *et al*., 1994), a finding in support of the presence of some virally encoded RNA *pol* subunits; however, it remains to be determined whether lef–8 alone is sufficient for regulated transcription *in situ* without the contribution of other subunits of either viral or host origin. Two alternate possibilities are that either a host polymerase is altered by subunit substitution, or the viral RNA *pol* subunits are unique and thus evade prediction based on database searches of putative ORFs.

Interactions with host cell cytoskeleton

Besides alterations noted in microfilament distribution at the time of virus entry, numerous changes to microfilament and microtubule distribution throughout infection have been documented (Charlton & Volkman, 1993b). The distribution of filamentous actin (F-actin) changes as viral replication proceeds, and is first manifested as transient cytoplasmic aggregates on the basal side of infected cells prior to viral DNA replication and dependant on early gene expression (Charlton & Volkman, 1991). After the initiation of viral DNA replication and gamma phase (late) gene expression F-actin was localized within the nucleus around the viral replication centre, the virogenic stroma, and in association with the major capsid protein, p39 (Charlton & Volkman, 1991). Treatment of infected cells with cytochalasin D (CD, 0.5–5 μg ml^{-1}) was found to dramatically affect viral morphogenesis, causing disruption of nucleocapsid formation so that only empty capsids were produced (Volkman *et al.*, 1987; Volkman, 1988; Hess *et al.*, 1989), and was correlated with altered distribution of actin to sites outside the nucleus (Volkman, 1988). CD has additional effects on NPV replication, including the delay of both host protein synthesis shutdown and expression of the hyperexpressed late (delta class) NPV genes encoding the p10 and polyhedrin proteins (Wei & Volkman, 1992); Oppenheimer & Volkman, 1995).

Microtubles alsoundergo significant changes as a result of baculovirus infection which may be responsible for the rounding of cells normally observed (Volkman & Zaal, 1990), and one of the first signs of viral CPE (Vaughn & Dougherty, 1985). Changes to the microtubule cytoskeleton are attributed to the action of both early and late viral genes (Volkman & Zaal, 1990). A hyperexpressed late viral protein (p10), which forms fibrillar masses in both cytoplasm and nucleus of infected cells (Vlak *et al.*, 1988; Williams *et al.*, 1989) associates with microtubules in the cytoplasm of infected cells, but is not attributed to the cytoskeletal changes (Volkman & Zaal, 1990). Interestingly, immunological cross-reaction between p10 and both cytoskeletal and nuclear components has been demonstrated (Quant-Russell *et al.*, 1987).

Host cell response to infection

Recently, a 35 kda viral protein of AcMNPV has been recognized as essential for successful viral infection in some insect cell lines but not others (Clem *et al.*, 1991). A mutant lacking this protein coding region (vAcAnh) could replicate in Tni cells, but induced cell death in Sf21 and *B. mori* (BmN–4) cells. In vAcAnh-infected Sf21 cells, progressive blebbing at the plasma membrane was seen to start at about 12 hr pi and increased in intensity, leading to premature cell death. Large membrane bound bodies were released from the dying cells although they contained morphologically and functionally intact mitochondria. Nuclei became fragmented and the chromatin was degraded into discrete fragments, hence apoptosis had been induced. Transient blebbing was seen in wt-infected Sf21 cells at 12 hr pi but cells progressed to form OB by 24 hr pi, and the cellular DNA remained in high molecular weight form. Blebbing was not seen in Tni cells infected with wt or vAcAnh, although virus stocks produced in these cells rapidly lost infectivity (L.K. Miller, personal communication).

A second apoptosis inhibiting protein, IAP (inhibitor of apoptosis, 31 kda), was identified in the genome of the *Cydia pomonella* granulosis virus, and its putative homologue in AcMNPV was also identified (Crook *et al.*, 1993; Ayres *et al.*, 1994). Both p35 and the granulosis-derived IAP were able to block induction of apoptosis by actinomycin D, but the IAP encoded by AcMNPV did not (Crook *et al.*, 1993). Insect cell apoptosis induced by actinomycin D could not be blocked by the mammalian suppressor BCL–2 (Cartier *et al.*, 1994), although BCL–2 was found to extend viablility of baculovirus-infected Sf21 cells and in particular prevent the internucleosomal DNA cleavage which normally occurs late in infection (Alnemri *et al.*, 1992). These results indicate some conservation in the cell death program of insect versus mammalian cells, and in support of this, baculovirus p35 was found to supress apoptosis in nematode and mammalian cells (Steller, 1995). Further sequence analysis of the AcMNPV genome revealed a second IAP-like coding region (IAP2, orf 71; Ayres *et al.*, 1994). The process of apoptosis is not well understood (for reviews see Bowen, 1993; Steller, 1995), but in the context of baculovirus replication it is clearly a host defense mechanism. It has been speculated that these baculovirus proteins (p35, IAP) may be involved in the shutdown of host protein and RNA synthesis (Clem *et al.*, 1991), as host transcription is required for apoptosis to occur.

Inhibition of host cell macromolecular synthesis

NPV infection is known to cause inhibition of both host DNA and protein synthesis (Vaughn & Dougher-

ty, 1985; Bilimoria, 1991) and by 8–12 hr pi only a small percentage of DNA synthesis is cellular. Studies using temperature sensitive mutants have demonstrated that in the absence of viral DNA replication, both host DNA synthesis (Brown *et al.*, 1979) and protein synthesis (Gordon & Carstens, 1984) are maintained. The shut down of host cell protein synthesis is considered slow (Kelly & Lescott, 1981) has been correlated with a reduction of host mRNA levels that occurs between 12–18 hr pi (Ooi & Miller, 1988); this latter study demonstrated that inhibition of viral DNA replication with aphidicolin also inhibited the reduction in host RNA levels. Because DNA replication is required for late but not early gene expression, it has been the consensus view that viral early genes do not play a direct role in host synthesis shut-off (Vaughn & Dougherty, 1985; Bilimoria, 1991). In consideration of the recent studies on the apoptosis blocking protein p35, the process of host shut-off may be considerably more complex and require the interplay of as yet undefined early and late gene functions.

Formation of the replication complex

The virogenic stroma and nucleocapsid morphogenesis

The first prominent change observed in infected cells is rounding of cells with concomittant enlargement of the nucleus (hypertrophy). Nuclear heterochromatin disappears and an intranuclear viral replication center known as the virogenic stroma is formed (Vaughn & Dougherty, 1985; Volkman & Knudson, 1986). These very early morphological events coincide with the start of gamma phase gene expression (late expression) with synthesis of structural proteins and vDNA. Several studies have noted coincident changes in nucleolar morphology (Tanada & Hess, 1976; Benz, 1986) with a transient increase in the abundance of RNA detected by histochemistry (Benz, 1986). Two temperature sensitive mutants have been described which are defective in late gene expression and cause disappearance of heterochromatin and nucleoli, but fail to form a defined stroma within most cells (Carstens *et al.*, 1994).

Involutions of the nuclear membrane morphologically similar to nucleolar canals are frequently seen at these early times post infection and may reflect high metabolic activity or inceased amount of nucleocytoplasmic exchange due to viral replication. Their appearance is transient, and may be correlated with the formation of the virogenic stroma. Infection of Sf cells by temperature sensitive mutants defective in late gene expression induced similar involutions of the nuclear membrane which later disintegrated, causing mitochondria to be located within the nucleus (Carstens *et al.*, 1994).

The virogenic stroma is considered a *de novo* product of baculovirus infection in which progeny virions are assembled. The stroma forms around the time vDNA replication is initiated (6–8 hr pi; Knudson & Harrap, 1976), and progeny virus particles appear in the stromal network shortly thereafter (about 10 hr pi). Initially the stroma appears as loose or dispersed throughout the nucleoplasm, but by 16–18 hr pi it condenses into a dense (mature) structure usually in the central region of the nucleus, giving rise to a peristromal ring zone in which intranuclear envelopment of virions and occlusion body formation take place. Two major regions are apparent in the mature stroma by electron microscopy: a fibrillar electron-dense matte forms the major "chromatic mass" and is interspersed with electron-lucent intrastromal spaces (Harrap, 1972b; Summers, 1971;Young *et al.*, 1993). The virogenic stroma was shown to be DNA-rich by Feulgen staining about forty years ago (Xeros, 1956). It also stains intensely with DNA-specific dyes such as DAPI or propidium iodide, and has a lattice-like appearance probably due to DNA distribution concentrated within the matte and not the intrastromal spaces. Because of the intensity and distinct pattern of DNA distribution in infected cells relative to uninfected controls, the single step DAPI-based fluorescent staining is a useful tool for determining the extent of infection independant of the presence of occlusion bodies, and may have potential for adaptation to a rapid $TCID_{50}$ assay.

Constituents of the stromal lattice are not well defined, although a recent study has indicated that in addition to DNA, a significant amount of RNA is present (Young *et al.*, 1993). Further, immunocytochemical studies in our laboratory demonstrated several NPV proteins and at least one cellular protein concentrated in the matte region (G.V. Williams, unpublished observations). Although the constituents of progeny nucleocapsids accumulate within the stromal matte, it is generally accepted that capsid shell formation and nucleocapsid maturation occurs in the intrastromal spaces (Harrap, 1972b; Fraser, 1986). Morphological and other data suggest that viral genomes are pre-packaged with the basic DNA-binding protein (p6.9) and the nucleoprotein complexes are subsequently inserted into the pre-formed cap-

sid shells. Assembly of the nucleoprotein core may be regulated by the state of phosphorylation of p6.9, which modulates its affinity for the viral DNA (Oppenheimer & Volkman, 1995; Funk & Consigli, 1993). The process of nucleocapsid maturation was shown to require intranuclear actin (Charlton & Volkman, 1991). In the presence of cytochalasin D packaging of viral DNA into the capsid shells was disrupted by proteolytic degradation of the unphosphorylated form of p6.9 (Oppenheimer & Volkman, 1995). Consistent with this model, there appear to be specialized regions within the stromal matte which are revealed by partial extraction of cells (Young *et al*., 1993) and by *en bloc* staining with tannic acid and uranyl acetate (G.V. Williams, unpublished observations). These regions are highly enriched with DNAse-sensitive filament bundles and were observed to be contiguous with the core of forming nucleocapsids in adjacent intrastromal spaces (Young *et al*., 1993). The sites are probably enriched with newly synthesised genomes accumulating prior to insertion into forming capsids.

DNA replication

Baculovirus DNA replication occurs within the virogenic stroma and is dependant on a virally encoded 126 kda DNA polymerase sensitive to aphidicolin (Wang & Kelly, 1983; Tomalski *et al*., 1988). The majority of the DNA replication machinery is considered to be virally encoded, although possible utilization of some host cell components is not excluded. Viral DNA synthesis starts 6–8 hr pi and continues for at least 12 hours, declining in rate by 18 hr pi (Tjia *et al*., 1979; Erlandson & Carstens, 1983). Few of the accessory genes required for DNA replication have been identified, although a non-essential proliferating cell nuclear antigen (PCNA) homologue, normally a component of DNA polymerase δ in eukaryotic cells, was identified in the genome of AcMNPV (Crawford & Miller, 1988; O'Reilly *et al*., 1989). Viral PCNA was not essential to DNA replication but deletion of part of the ORF caused a delay in all late viral gene expression, and slowed progression of infection in insect larvae. Another viral gene, designated p143, has 7 regions of homology to known helicase proteins (Lu & Carstens, 1992a), and a temperature sensitive lesion within this gene caused a DNA replication defective condition (Lu & Carstens, 1991). Recently a transient complementation assay was used to identify essential *trans*-acting factors required for plasmid DNA replication in insect cells. Cotransfection of cosmid clones in a subtractive assay led to the identification of 6 genome regions encoding at least 7 genes essential to viral DNA replication (Kool *et al*., 1994a). This assay confirmed the absolute necessity of the p143 (helicase) and p126 (DNA Pol) genes but not the vPCNA gene for viral DNA replication. Further analysis indicated that the viral genes encoding ie–1 and late expression factors (lef) 1, 2, and 3 were also essential, and viral DNA replication was stimulated by the p35, ie–2 (formerly ie-n), and pe38 proteins (Kool *et al*., 1994b). The putative ORI (HR) sequences were not essential for plasmid replication in the presence of transfected cosmid clones (Kool *et al*., 1994a), but were essential in experiments that used whole virus as the source of *trans*-acting factors (Kool *et al*., 1993). Viral DNA replication normally occurs within the virogenic stroma in baculovirus infected cells (Volkman & Keddie, 1990) and is concommitant with morphogenesis of the structure (Knudson & Harrap, 1976), so the HRs may be essential for efficient DNA replication within the stroma. Comparison of NPV DNA replication proteins with DNA replication proteins of *Herpesviridae* has indicated possible homology between these virus families, and both seem to share a core set of 6 essential genes. Based on this study, amino acid sequence alignments suggest that ie–1 codes for a single stranded DNA binding protein, and lef 1–3 code for a primase-associated protein, a DNA polymerase processivity factor, and a primase, respectively (Kool *et al*., 1994b).

Late processes in morphogenesis

Virion phenotypes and biphasic replication

Intrastromal spaces communicate with the surrounding nucleoplasm of the ring zone, hence mature nucleocapsids are able to migrate into this peristromal compartment. Nucleocapsids destined to be components of BVegress from the nucleus at about 24 hr pi (Fraser, 1986; Kawamoto *et al*., 1977). Later, BV production is curtailed by an undefined mechanism in favour of intranuclear envelopment of nucleocapsids (Figure 1; Volkman *et al*., 1976; Stoltz *et al*., 1973; Tanada & Hess, 1976). Nucleocapsids of both phenotypes are seen to align with and attach to membranes at the apical (nipple) end of the capsid (Kawamoto *et al*., 1977; Fraser, 1986). Several methods of nucleocapsid egress from the nucleus have been suggested, including exit via nuclear pores (Hess & Falcon, 1977; Summers, 1971), migration into or through the cellular endomem-

brane systm, or passage through discontinuities in the nuclear membrane (Adams *et al*., 1977). Of interest is the apparent loss of the nuclear membrane-derived vesicle in transit, as naked capsids are predominant at sites of budding along the plasma membrane. No model for the release of capsids from transport vesicles has been proposed; the process may involve fusion or disintegration of membranes. Cytoplasmic extrusion of nuclear membrane vesicles is not strictly dependant on the presence of capsids, as mutants defective in late gene expression commonly induced budding of the nuclear membrane independant of capsid formation (Carstens *et al*., 1994). Because some late protein synthesis did occur,some capsid components, in particularthose proteins associated with the apical cap strucuture, may have induced the extrusion phenomenon.

Glycosylation is an important process in the morphogenesis of mature budded virions. Kelly & Lescott (1983) reported that *Tni*MNPV grown in *Sf* cells required N-glycosylation for proper envelopment of virions, and in the presence of tunicamycin nucleocapsids accumulated in the cytoplasm of drug-treated cells and budding at the plasma membrane was reduced or eliminated. A modest dose of tunicamycin ($10\ \mu g\ ml^{-1}$) caused a 90% reduction in virus titre. In a similar study in which AcMNPV replicated in *Tni* cells there was again a decline inproduction of infectious virus in the presence of $10\ \mu g\ ml^{-1}$ tunicamycin (Stiles *et al*., 1983). Progeny virus was morphologically indistinguishable from normal BV although they were of lower buoyant density.

Switchover from BV to PDV phenotype morphogenesis

The intranuclear ring zone is site of several morphogenic processes closely associated with OB development late in infection, including intranuclear envelopment and occlusion of virions, condensation of polyhedrin into paracrystalline arrays and finally the attachment of the occlusion body calyx. The mechanism of switchover from BV to occluded virus is not known, and although BV production is reduced substantially in favour of intranuclear envelopment some budding at the plasma membrane is observed late in infection (48 hr pi). Concommitant with the decrease in BV production, patches of membranous profiles and vesicles develop within the nuclear ring zone which are precursor structures utilized for intranuclear envelopment of virions. Based on the distinct dimensions and trilamellar morphology of the membranous profiles, and apparent lack of continuity with the nuclear membrane, these were postulated to be the result of *de novo* synthesis (Stoltz *et al*., 1973). Baculovirus infection has been observed to induce an invagination of the inner nuclear membrane (Tanada & Hess, 1976; Summers & Arnott, 1969), and this may be the source material for the intranuclear membrane vesicles.

Immunocytochemical localization studies have indicated that a 66 kda structural protein associated with the PDV envelope (PDV-E66) co-localized to these intranuclear membrane patches but not nuclear membrane (Hong *et al*., 1994). A 25 kda PDV envelope protein of OpMNPV was found to localize to virions within the ring zone and within mature OB (Russell & Rohrmann, 1993), and was also identified at the periphery of the virogenic stroma. The major tegument glycoprotein exclusive to the occluded virion phenotype (gp41) has a similar distribution (G.V. Williams, unpublished observations), as does a capsid end structure-associated protein (p78) found in both viral phenotypes (Vialard & Richardson, 1993). The most likely explanation for these observations is that nucleocapsids leaving the virogenic stroma are intercepted by molecules necessary for completion of morphogenesis, and regulation of peri-stromal accumulation might govern the switchover from budded to occluded virion phenotypes.

OB morphogenesis

OB formation occurs by polyhedrin condensation within the intranuclear ring zone of infected cells and is well established by 24 hr pi (Volkman & Knudson, 1986). The paracrystalline arrays are described as a face-centered cubic lattice comprised of 4–5 nm subunits packed in a rhombic arrangement (Federici, 1986; Kelly, 1985; Harrap, 1972a). Virions within the matrix are randomly distributed and do not interfere with the regularity of the lattice (Harrap, 1972a; Harrap & Payne, 1979). Harrap (1972a) examined polyhedrin crystals of partially dissolved OB by TEM using a negative stain procedure, and determined that polyhedrin oligomers form a six nodal structure. The size and shape of OBs are heritable features (Gershenson, 1960) and are intimately related to the primary sequence of polyhedrin (Brown *et al*., 1980; Carstens *et al*., 1986; Carstens *et al*., 1992). Alterations in the polyhedrin coding region cause dramatic alterations in the form of the paracrystalline lattice (Brown *et al*., 1980; Duncan & Faulkner, 1982; Carstens *et al*., 1986, 1987, 1992) and usually abrogate the occlusion of virions, but no clear peptide

domain or signal motif has been associated with virion incorporation.

Polyhedrin (29 kda) is a virus encoded protein having 245 amino acid residues (Summers & Smith, 1976; Rohrmann, 1986; Rohrmann, 1992); it possesses a nuclear localization motif (Jarvis *et al.*, 1991) which facilitates accumulation in the ring zone. Post-translational modification of polyhedrin includes phosphorylation (Maruniak & Summers, 1981; Dobos & Cochran, 1980), and possibly glycosylation (Kelly & Lescott, 1983), but the functional significance of these post-translational modifications is not known. Polyhedrin and another hyperexpressed late gene product, p10, are detected at about 12 hr pi in AcMNPV infected cells (Rohel *et al.*, 1983) and production continues throughout the replication cycle. The peptide p10 is not a structural component of virions or OB, but forms large fibrillar masses which are intimately associated with maturing OB (Vlak *et al.*, 1988; Williams *et al.*, 1989); thus some p10 may copurify with the occlusions (Vlak *et al.*, 1981). Morphogenesis of fibrillar bodies, occlusion bodies, and the OB calyx seems to occur independantly, as deletion mutants which affect one of the structures do not affect formation of the other structures (van der Wilk, 1987; Williams *et al.*, 1989; Zuidema *et al.*, 1989), nor do these same mutants seem to affect the intranuclear envelopment of virions.

In addition to the occluded virions several organic and inorganic factors may also be incorporated intothe OB matrix. A viral enhancing factor (VEF) in GV and NPV occlusions which acts on the host insect peritrophic has been identified (Derksen & Granados, 1988). As well, OB derived from insects may contain a significant amount of silicon (Stairs, 1968) which may be incorporated into the lattice, but the distribution has not been determined nor have tissue culture derived OB been examined for inorganic components. There are also several accounts of the presence of RNA in purified OB (Faulkner, 1962; Aizawa & Tida, 1963; Himeno *et al.*, 1969) in approximately equal quantity relative to viral DNA (Faulkner, 1962). These molecules and inorganic components have not been localized within the OB structure, and may be associated with embedded virions, dispersed throughout the occlusion matrix, or copurified as contaminants at the surface of the OB.

Regulatory signal(s) which govern cessation of crystal growth and attachment of calyx is (are) not known, and the process may be mediated in a temporal fashion by specific enzymes or may be inherent to the crystalline structure. At least one polyhedrin mutant displayed greatly increased affinity for calyx precursor (Carstens *et al.*, 1992), suggesting that a conformational shift in the polyhedrin molecule or oligomeric superstructures may be involved in this process.

Calyx morphogenesis and attachment

The calyx is an electron dense carbohydrate-rich structure (Minion *et al.*, 1979; Whitt & Manning, 1988), and has also been called the OB membrane (Mackinnon *et al.*, 1974; Harrap, 1972a) and OB envelope (Longworth *et al.*, 1972). A TEM study of the calyx by negative staining indicated a regular array distinct from the polyhedrin lattice, with 15 nm holes or hollows evenly distributed across the structure (Harrap, 1972a). After release of PDV from occlusions by alkali dissolution the calyx remains as a residual bag-like structure (Harrap, 1972a).

The ontogeny of the calyx is unknown and is considered a *de novo* virus structure which forms as bilamellar sheet precursors in association with intranuclear p10 fibrillar bodies (MacKinnon *et al.*, 1974; Van der Wilk *et al.*, 1987). Calyx precursor formation is not dependant on the presence of p10 (Williams *et al.*, 1989), but this association is important for proper attachment to maturing OB, and deletion of the p10 gene adversely affects the stability of the OB to withstand physical stress (Williams *et al.*, 1989). A 34 kda phosphoprotein (pp34; Gombart *et al.*, 1989) has been immunolocalized to the calyx (Russell & Rohrmann, 1990a; van Lent *et al.*, 1990) and is covalently associated by thiol bonds (Whitt & Manning, 1988). A subpopulation of polyhedrin is also thiol linked to the calyx (Whitt & Manning, 1988) which may anchor this structure to the surface of the OB, although the specific interactions between calyx, pp34, and polyhedrin have not been analysed. Deletion mutants lacking the pp34 protein fail to produce a calyx, and the OB product has increased sensitivity to alkali (Zuidema *et al.*, 1989; Vlak *et al.*, 1988). A 34.8 kda spheroidin-like protein (SLP; Vialard *et al.*, 1990) has also been localized to the OB of AcMNPV and may be a component of the calyx (Rohrmann, 1992), but its role in calyx morphogenesis or structure is not known, and in OpMNPV the putative SLP does not seem calyx associated (Rohrmann, 1992).

Few polyhedra (FP) mutants

Distinct from mutations within the polyhedrin coding region, several mutants of AcMNPV have been charac-

terized which also exhibit OB production manifested as the few polyhedra (FP) phenotype. FP mutants arise with high frequency in serially passaged NPV in tissue culture (Potter *et al.*, 1976) and the resulting OB contain few if any occluded virions (Potter *et al.*, 1976; Ramoska & Hink, 1974). The genetic defect is generally attributed to loss of a PDV-associated 25 kda protein (Fraser *et al.*, 1983; Beames & Summers, 1989) and results in the production of fewer than 10 OB/cell. The p25 gene codes for an envelope protein of the PDV phenotype and appears to be essential for envelopment of intranuclear virions (Harrison & Summers, in press). The protein could be an important factor for initiation of OB formation, perhaps by causing nucleation of polyhedrin (Wood, 1980); alternately, completely enveloped intranuclear virions may trigger OB formation. The p25 gene is not exclusively responsible for the FP phenotype because FP mutants were are also generated by alteration of an early gene, p94 (Miller & Miller, 1982; Friesen & Miller, 1987).

Cytolysis and OB release

Late in AcMNPV and SeMNPV infection, after OB production and maturation, a nuclear disintegration event occurs which facilitates the release of OB (Williams *et al.*, 1989; van Oers *et al.*, 1993). This process is dependant on the presence of p10 (Williams *et al.*, 1989) and the function has been mapped between amino acids 52–79 in the case of AcMNPV (van Oers *et al.*, 1993). The mechanism and other potential components involved in cytolysis and OB release are not known. It was originally reported that release occurred in both Sf and Tni cell lines starting at about 60 hr pi (Williams *et al.*, 1989). Recently we observed that Sf 21 and Sf9 cells cultured in our laboratory no longer support release although normal cytolysis and release of OB was observed in Tni cells (G.V.Williams, unpublished observations). Preliminary experiments have ruled out the cell or virus passage history and we currently attribute the phenomenon to subtle alterations in components of the cell culture media.

Conclusion

In vitro studies have contributed greatly to an understanding of viral cytopathology, molecular biology, and pathogenesis. A model of the role of baculoviruses in a host-parasite relationship is developing which reveals the virus as gaining control of many aspects of host cell biology including control of the cell replication machinery (apoptotic response, macromolecular synthesis), the cytoskeletal structure, the nuclear membrane and intranuclear architecture. Baculovirus replication is a collection of independent but inter-related processes which work within the framework of the host cell, with the *in vivo* goal of maximizing production of progeny virions. Further molecular dissection of baculovirus replication should yield insight into the processes and principles of viral and host regulatory systems, perhaps facilitating development of new generations of high efficiency sub-viral expression vector systems and the development of genetically improved strains of virus safe for field use in ecologically based pest management strategies.

References

Adams JR, Goodwin RH & Wilcox TA (1977) Electron microscopic investigations on invasion and replication of insect baculoviruses *in vivo* and *in vitro.* Biologie Cellulaire 28: 261–268.

Aizawa K & Tida S (1963) Nucleic acids extracted from the virus polyhedra of the silkworm, *Bombyx mori* (Linnaeus). Journal of Insect Pathology 5: 344–348.

Alnemri, ES Robertson, NM Fernandes, TF Croce, CM & Litwack, G (1992) Overexpressed full-length human BCL2 extends the survival of baculovirus-infected Sf9 insect cells. Proceeding of the National Academy of Sciences USA 89: 7295–7299.

Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M & Possee RD (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202: 586–605

Bassemir U, Miltenburger HG & David P (1983) Morphogenesis of nuclear polyhedrosis virus from Autographa californica in a cell line from Mamestra brassicae (Cabbage moth). Further aspects of baculovirus assembly. Cell and Tissue Research 228: 587–595

Beames B & Summers MD (1989) Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants. Virology 168: 344–353.

Benz GA (1986) Introduction: Historical perspectives. In: Granados RR & Federici BA (eds) The biology of baculoviruses. CRC Press, Inc, Boca Raton, pp. 1–35

Bilimoria SL (1991) The biology of nuclear polyhedrosis viruses. In: Kurstak E (ed) Viruses of Invertebrates. Marcel Dekker, New York, pp. 1–72

Bjornson RM & Rohrmann GF (1992) Nucleotide sequence of the polyhedron envelope protein gene region of the *Lymantria dispar* nuclear polyhedrosis virus. Journal of General Virology 73: 1499–1504

Blissard GW, Quant-Russell RL, Rohrmann GF & Beaudreau GS (1989) Nucleotide sequence, transcriptional mapping and temporal expression of the gene encoding p39 a major structural protein of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata.* Virology 168: 354–362

Blissard GW & Rohrmann GF (1989) Location, sequence, transcriptional mapping and temporal expression of the gp64 gene of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology 170: 537–555.

Blissard GW & Rohrmann GF (1990) Baculovirus diversity and molecular biology. Annual Review of Entomology 35: 127–155.

Bowen ID (1993) Apoptosis or programmed cell death? Cell Biology International 17: 365–380.

Braunagel SC & Summers MD (1994) *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: Structural proteins, antigens, lipid and fatty acid profiles. Virology 202: 315–328.

Brown M, Crawford AM & Faulkner P (1979) Genetic analysis of a baculovirus, *Autographa californica* nuclear polyhedrosis virus. I.Isolation of temperature sensitive mutants and assortment into complementation groups. Journal of Virology 31: 190–198.

Brown M, Faulkner P, Cochran MA & Chung KL (1980) Characterization of two morphology mutants *of Autographa californica* nuclear polyhedrosis virus with large cuboidal inclusion bodies. Journal of General Virology 50: 309–316.

Burand JP, Summers MD & Smith G (1980) Transfection with baculovirus DNA. Virology 101: 286–290.

Carstens EB, Chan H, Yu H, Williams GV & Casselman R (1994) Genetic analysis of temperature-sensitive mutations in baculovirus late exprssion factors. Virology 204: 323–337

Carstens EB, Tjia ST & Doerfler W (1980) Infectious DNA from *Autographa californica* nuclear polyhedrosis virus. Virology 101: 311–314

Carstens EB, Krebs A & Gallerneault CE (1986) Identification of an amino acid essential to the normal assembly *of Autographa californica* nuclear polyhedrosis virus polyhedra. Journal of Virology 58: 684–688.

Carstens EB, Williams GV, Faulkner P & Partington S (1992) Analysis of polyhedra morphology mutants of *Autographa californica* nuclear polyhedrosis virus: Molecular and ultrastructural features. Journal of General Virology 71: 3035–3040.

Carstens EB, Ye L-B & Faulkner P (1987) A point mutation in the polyhedrin gene of a baculovirus, *Autographa californica* MNPV, prevents crystallization of occlusion bodies. Journal of General Virology 68: 901–905.

Cartier JL, Hershberger PA & Friesen PD (1994) Suppression of apoptosis in insect cells stably transfected with baculovirus p35: Dominant interference by N-terminal sequences $p35^{1-76}$. Journal of Virology 68: 7728–7737.

Charlton CA & Volkman LE (1991) Sequential rearrangement and nuclear polymerization of actin in baculovirus-infected *Spodoptera frugiperda* cells. Journal of Virology. 65: 1219–1227.

Charlton CA & Volkman LE (1993a) Penetration of *Autographa californica* nuclear polyhedrosis virus nucleocapsids into IPLB Sf–21 cells induces actin cable formation. Virology 197: 245–254.

Charlton CA & Volkman LE (1993b) Baculoviruses, vertebrate viruses and cytoskeletons. In: "Parasites and Pathogens of Insects" Edited by Beckage NE, Thompson SN & Federici BA (eds), pp. 103–125. Academic Press, New York.

Chirwin JM, Przbyla AE, MacDonald RJ & Rutter WJ (1979) Isolation of biologically active RNA from sources enriched in ribonuclease. Biochemistry 18: 5294–5299.

Clem RJ, Fechheimer M & Miller LK (1991) Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science 254: 1388–1390.

Crawford AM & Miller LK (1988) Characterization of an early gene accelerating expression of late genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 62: 2773–2781.

Crook NE, Clem R & Miller LK (1993) An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. Journal of Virology 67: 2168–2174.

Derksen ACG & Granados RR (1988) Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology 167: 242–250.

Dobos P & Cochran MA (1980) Protein synthesis in cells infected by *Autographa californica* nuclear polyhedrosis virus (AcNPV): The effect of cytosine arabinoside. Virology 103: 446–464

Duncan R & Faulkner P (1982) Bromodeoxyuridine-induced mutants of *Autographa californica* nuclear polyhedrosis virus defective in occlusion body formation. Journal of General Virology 62: 369–373.

Erlandson MA & Carstens EB (1983) Mapping early transcription products of *Autographa californica* nuclear polyhedrosis virus. Virology 126: 398–402.

Faulkner P, Kuzio J & Williams GV (1995) Analysis of p74, a protein of *Autographa californica* nuclear polyhedrosis virus required for occlusion body infectivity *in vivo.* Journal of General Microbiology, (in press).

Faulkner P (1981) Baculovirus. In: Davidson EW (ed.) Pathogenesis of Invertebrate Microbial Disease Allenhead,Osmun and Co. Totowa, New Jersey, pp. 3–33

Faulkner P (1962) Isolation and analysis of ribonucleic acid from inclusion bodies of the nuclear polyhedrosis of the silkworm. Virology 16: 479–484.

Federici BA (1986) Ultrastructure of Baculoviruses. In: Granados RR & Federici BA (eds) The biology of baculoviruses. CRC Press, Inc, Boca Raton, pp. 61–88

Francki RIB, Fauquet CM, Knudson DL & Brown F (1991) Classification and nomenclature of viruses. Archives of Virology Supplementum 2, pp. 117–123.

Fraser MJ (1986) Ultrastructural observations of virion maturation in *Autographa californica* nuclear polyhedrosis virus infected *Spodoptera frugiperda* cell cultures. Journal of Ultrastructure and Molecular Structure Research 95: 189–195.

Fraser MJ, Smith GE & Summers MD (1983) Aquisition of host-cell DNA sequences by baculoviruses relationship between host DNA insertions and FP mutants of*Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. Journal of Virology 47: 287–300.

Friesen PD & Miller LK (1987) Divergent transcription of early 35- and 94 kilodalton protein genes encoded by the HindIII K genome fragment of the baculovirus *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 61: 2264–2272

Friesen PD & Miller LK (1985) Temporal regulation of baculovirus RNA: overlapping early and late transcripts. Journal of Virology 54: 392–400.

Friesen PD & Miller LK (1986) The Regulation of Baculovirus Gene Expression. In *Current Topics in Microbiology and Immunology.* Doerfler W & Böhm, P (eds). 131: 31–49. Springer-Verlag, Berlin.

Fuchs LY, Woods MS & Weaver RF (1983) Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: A novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. Journal of Virology 48: 641–646.

Funk CJ & Consigli RA (1993) Phosphate cycling on the basic protein of *Plodia interpunctella* granulosis virus. Virology 193: 396–402.

Gershenson SM (1960) A study of a mutant strain of nuclear polyhedral virus of oak silkworm. Problems of Virology (English translation of *Voprosy virusologii)* 6: 720–725.

Gombart AF, Pearson MN, Rohrmann GF & Beaudreau GS (1989b) A baculovirus polyhedral envelope-associated protein: Genetic location, nucleotide sequence, and immunocytochemical characterization. Virology 169: 182–193.

Gordon JD & Carstens EB (1984) Phenotypic characterization and physical mapping of a temperature sesitive mutant *of Autographa californica* nuclear polyhedrosis virus defective in DNA synthesis. Virology 138: 69–81

Granados RR (1978) Early events in the infection of *Heliothis zea* midgut cells by a baculovirus. Virology 90: 170–174

Granados RR & Lawler KA (1981) *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. Virology 108: 297–308.

Granados RR & Williams KA (1986) *In vivo* infection and replication of baculoviruses In: Granados RR & Federici BA (eds) The , biology of baculoviruses. CRC Press, Inc, Boca Raton, pp. 89–108.

Grula MA, Buller PL & Weaver RF (1981) Alpha-Amanitin resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. Journal of Virology 38: 916–921.

Harrap KA (1972a) The Structure of Nuclear Polyhedrosis Viruses. I. The Inclusion Body. Virology 50: 114–123.

Harrap KA (1972b) The Structure of Nuclear Polyhedrosis Viruses. III. Virus Assembly. Virology 50: 133–139.

Harrap KA & Payne CC (1979) The structural properties and identification of insect viruses. Advances in Virus Research 25: 273–355.

Harris H (1987) Microfilament dynamics. Few answers but many questions. Nature 330: 310–311.

Harrison & Summers MD (1995) Mutations in *the Autographa californica* nuclear polyhedrosis virus 25K gene cause the entire range of the "few polyhedra" phenotype. Journal of General Virology (in press).

Hess RT & Falcon LA (1977) Observations on the interaction of baculoviruses with the plasma membrane. Journal of General Virology 36: 525–530.

Hess RT, Goldsmith PA & Volkman LE (1989) Effect of cytochalasin D on cell morphology and AcMNPV replication in a *Spodoptera frugiperda* cell line. Journal of Invertebrate Pathology 53: 169–182.

Himeno M, Yasuda S, Kohsaka T & Onodera K (1969) The fine structure of a nuclear-polyhedrosis virus of the silkworm. Journal of Invertebrate Pathology 13: 87.

Hink WF (1982) Production *of Autographa californica* nuclear polyhedrosis virus in cells from large scale suspension cultures. In: Kurstak E (ed.) Microbial and vira pesticides. Marcel Dekker, New York, pp. 493–506.

Hirokawa N (1994). Microtubule organization and dynamics dependent on microtubule-associated proteins. Current Opinion in Cell Biology 6: 74–81.

Hohmann AW & Faulkner P (1983). Monoclonal antibodies to baculovirus structural proteins: determination of specificities by western blot analysis. Virology 125: 432–444.

Hong T, Braunagel SC & Summers MD (1994) Transcription,translation, and cellular localization of PDV-E66: A structural protein of the PDV envelope of *Autographa californica* nuclear polyhedrosis virus. Virology 204: 210–222.

Hoopes RR & Rohrmann GF (1991) In vitro transcription of baculovirus immediate early genes: Accurate mRNA initiation by nuclear extracts from both insect and human cells. Proceedings of the National Academy of Sciences USA 88: 4513–4517.

Horton HM & Burand JP (1993) Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. Journal of Virology 67: 1860–1868.

Huh NE & Weaver RF (1990a) Categorizing some early and late transcripts directed by the *Autographa californica* nuclear polyhedrosis virus. Journal of General Virology 71: 2195–2200.

Huh NE & Weaver RF (1990b) Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. Journal of General Virology 71: 195–201.

Jarvis DL, Bohlmeyer DA & Garcia A Jr (1991) Requirements for nuclear localization and supramolecular assembly of a baculovirus polyhedrin protein Virology 185: 795–810.

Jaques RP(1977) Stability of entomopathogenic viruses. Misc. Publ. Entomol. Soc. Amer. 10: 99–117.

Jaques RP (1985) Stability of insect viruses in the environment. In: Maramorosch, K & Sherman, KE (eds) Viral Insecticides for Biological Control. Academic Press, Inc, New York, pp. 285–360.

Kawamoto F, Suto C, Kumada N & Kobayashi M (1977). Cytoplasmic budding of a nuclear polyhedrosis virus and comparative ultrastructural studies of envelopes. Microbiol Immunol 21: 255–265.

Kawanishi CY, Summers MD, Stoltz DB & Arnott HJ (1972) Entry of an insect virus in vivo by fusion of viral envelope and microvillus membrane. Journal of Invertebrate Pathology 20: 104–108.

Keddie BA, Apointe GW & Volkman LE (1989) The pathway of infection *of Autographa californica* nuclear polyhedrosis virus in an insect host. Science 242: 1728–1730.

Keddie BA & Volkman LE (1985) Infectivity difference between the two phenotypes *of Autographa californica* nuclear polyhedrosis virus: Importance of the 64K envelope glycoprotein. Journal of General Virology. 66: 1195–2000.

Kelly DC (1985) The structure and physical characteristics of Baculoviruses. In *Viral Insecticides for Biological Control.* Edited by K Maramorosch & KE Sherman. pp. 469–488. Academic Press Inc New York.

Kelly DC, Brown DA, Ayres MD, Alien CJ & Walker IO (1983) Properties of the major nucleocapsid protein of *Heliothis zea* singly enveloped nuclear polyhedrosis virus. Journal of General Virology. 64: 399–408.

Kelly DC & Lescott T (1981) Baculovirus replication: Protein synthesis in *Spodoptera frugiperda* cells infected with *Trichoplusia ni* nuclear polyhedrosis virus. Microbiologica 4: 35–57.

Kelly DC & Lescott T (1983) Baculovirus replication: Glycosylation of polypeptides synthesized in *Trichplusia ni* nuclear polyhedrosis virus infected cells and the effect of tunicamycin. Journal of General Virology 64: 1915–1926.

Kelly DC & Wang X (1981) The infectivity of nuclear polyhedrosis virus DNA. Ann. Virol. (Inst Pasteur) 132E: 247–259.

Knudson DL & Harrap KA (1976) Replication of a nuclear polyhedrosis virus in a continuous cell culture of *Spodopterafrugiperda:* Microscopy study of the sequence of events of the virus infection. Journal of Virology 17: 254–268.

Kool M, Voeten JTM, Goldbach RW, Tramper J & Vlak JM (1993) Identification of seven putative origins *of Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus DNA replication. Journal of General Virology 74: 2661–2668.

Kool M, Voeten JTM, Goldbach RW & Vlak JM (1994a) Functional mapping of regions of the *Autographa californica* nuclear polyhedrosis viral genome required for DNA replication. Virology 198: 680–689.

Kool M, Ahrens CH, Goldbach RW, Rohrmann GF *&* Vlak JM (1994b) Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proceedings of the National Academy of Sciences, USA. In Press.

Kuzio J, Jaques R & Faulkner P (1989) Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. Virology 173: 759–763.

Longworth JF, Robertson JS & Payne CC (1972) The purification and properties of inclusion body protein of the granulosis virus *Pieris brassicae.* Journal of Invertebrate Pathology 19: 42–50.

Lu A & Carstens EB (1991). Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis virus. Virology 181: 336–347.

Lu A & Carstens EB (1992a) Transcription analysis of the EcoRI-D region of the baculovirus *Autographa californica* nuclear polyhedrosis virus identifies an early 4-kilobase RNA encoding the essential p143 gene. Journal of Virology 66: 655–663.

Lu A & Carstens EB (1992b) Nucleotide sequence and transcriptional analysis of the p80 gene of *Autographa californica* nuclear polyhedrosis virus: a homologue of the *Orgyia pseudotsugata* nuclear polyhedrosis virus capsid-associated gene. Virology 190: 201–209.

Mackinnon EA, Henderson JF, Stoltz DB & Faulkner P (1974) Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro.* Journal of Ultrastructural Research 49: 419–435.

Maeda S, Karnita SG & Kataoka H (1991) The basic DNA-binding protein of *Bombyx mori* nuclear polyhedrosis virus: The existence of an additional arginine repeat. Virology 180: 807–810.

Maruniak JE & Summers MD (1981) *Autographa californica* nuclear polyhedrosis virus phosphoproteins and synthesis of intracellular proteins after virus infection. Virology 109: 25–34.

Miller LK (1989) Insect baculoviruses: Powerful gene expression vectors. BioEssays 11: 91–95.

Miller LK, Adang MJ & Browne D (1983) Protein kinase activity associated with the extracellular and occluded forms of the baculovirus *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 46: 275–278.

Miller DW & Miller LK (1982) A virus mutant with an insertion of a copia-like transposable element. Nature (London) 299: 562–564.

Minion FC, Coons LB & Broome JR (1979) Characterization of the polyhedral envelope of the nuclear polyhedrosis virus of *Heliothis virescens.* Journal of Invertebrate Pathology. 34: 303–307.

Muller R, Pearson MN, Russell RLQ & Rohrmann GF (1990) A capsid-associated protein of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata*: Genetic location, sequence, transcriptional mapping, and immunocytochemical characterization. Virology 176: 133–144.

Nagata M & Tanada Y (1983) Origin of an alkaline protease associated with the capsule of a granulosis virus of the armyworm, *Pseudaletia unipuncta* (Haworth). Archives of Virology. 76: 245–256.

Ooi BG & Miller LK (1988) Regulation of host RNA levels during baculovirus infection. Virology 166: 515–523.

Oppenheimer DI & Volkman LE(1995) Proteolysis of p6.9 induced by cytochalasin D in *Autographa californica* M nuclear polyhedrosis virus-infected cells. Virology 207: 1–11.

O'Reilly DR, Miller LK & Luckow VA (1992) Baculovirus expression vectors. WH Freeman and Company, New York.

O'Reilly DR, Crawford AM & Miller LK (1989) Viral proliferating cell nuclear antigen. Nature 337: 606.

Passarelli AL, Todd JW & Miller LK (1994) A baculovirus gene involved in transient late gene expression encodes a 102- kilodalton polypeptide with a motif conserved in RNA polymerases. (Sequence in EMBL/Genebank Acc. No. U04879).

Pearson MN, Russell RQ, Rohrmann GF & Beaudreau GS (1988) p39, A major baculovirus structural protein: Immunocytochemical characterization and genetic location Virology 167: 407–413.

Possee RD, Sun T-P, Howard SC, Ayres MD, Hill-Perkins M & Gearing KL (1991) Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis 9.4 kbp *Eco*RI-I and -R (polyhedrin gene) region. Virology 185: 229–241.

Potter KN, Faulkner P & Mackinnon EA (1976) Strain selection during serial passage of *Trichoplusia ni* nuclear polyhedrosis virus. Journal of Virology 18: 1040–1050.

Potter KN & Miller LK (1980) Transfection of two invertebrate cell lines with DNA of *Autographa californica* nuclear polyhedrosis virus. Journal of Invertebrate Pathology 36: 431–439.

Quant-Russell RL, Pearson MN, Rohrmann GF & Beaudreau GS (1987) Characterization of baculovirus p10 synthesis using monoclonal antibodies. Virology 160: 9–19.

Ramoska WA & Hink WF (1974) Electron microscope examination of two plaque variants from a nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica.* Journal of Invertebrate Pathology 23: 197–201.

Roberts TE & Faulkner P (1989) Fatty acid acylation of the p67 glycoprotein of a baculovirus: *Autographa californica* nuclear polyhedrosis virus. Virology 172: 377–381.

Rohel DZ, Cochran MA & Faulkner P (1983) Characterization of two abundant mRNAs of *Autographa californica* nuclear polyhedrosis virus present late in infection. Virology 124: 357–365.

Rohrmann GF (1986) Polyhedrin structure. Journal of General Virology 67: 1499–1513.

Rohrmann GF (1992) Baculovirus structural proteins. Journal of General Virology 73: 749–761.

Rubenstein R & Polson A (1983) Midgut and viral associated proteases of *Heliothis armigera.* Intervirology 19: 16–25.

Russell RLQ, Pearson MN & Rohrmann GF (1991) Immunoelectron microscopic examination of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus-infected *Lymantria dispar* cells: Time course and localization of major polyhedron-associated proteins. Journal of General Virology 72: 275–283.

Russell RLQ & Rohrmann GF (1990) A baculovirus polyhedron envelope protein: Immunogold localization in infected cells and mature polyhedra. Virology 174: 177–184.

Russell RLQ & Rohrmann GF (1991) The p6.5 gene region of a nuclear polyhedrosis virus in *Orgyia pseudotsugata*: DNA sequence and transcription analysis of four late genes. Journal of General Virology 71: 551–560.

Russell RLQ & Rohrmann GF (1993) A 25-kDa protein is associated with the envelopes of occluded baculovirus virions. Virology 195: 532–540.

Schetter C, Oellig C & Doerfler W (1990). An insertion of insect cell DNA in the 81-map-unit segment of *Autographa californica* nuclear polyhedrosis virus DNA. Journal of Virology 64: 1844–1850.

Slack JM, Kuzio J & Faulkner P (1995) Characterization of *v-cath*, aCathepsin L-like proteinase expressed by the baculovirus AcMNPV Journal of General Virology

Stairs GR (1968) Inclusion-Type Insect Viruses. In: *Current Topics in Microbiology and Immunology* 42, 1–23. Maramorosch K (ed). Springer-Verlag New York Inc.

Steller H (1995) Mechanisms and genes of cellular suicide. Science 267: 1445–1449.

Stiles B, Wood HA & Hughes PR (1983) Effect of tunicamycin on the infectivity of *Autographa californica* nuclear polyhedrosis virus. Journal of Invertebrate Pathology 41: 405–408.

Stiles B & Wood HA (1983) A study of the glycoproteins of *Autographa californica* nuclear polyhedrosis virus (AcNPV). Virology 131: 230–241.

Stoltz DB, Pavan C & DaCunha AB (1973) Nuclear polyhedrosis virus: A possible example of *de novo* intranuclear membrane morphogenesis. Journal of General Virology 19: 145–150.

Summers MD & Arnott HJ (1969) Ultrasturctural studies on inclusion formation and virus occlusion in nuclear polyhedrosis and granulosis virus-infected *Trichoplusia ni* (Hübner). Journal of Ultrastructural Research. 28: 462–480.

Summers MD & Smith GE (1976) Comparative studies of baculovirus granulins and polyhedrins. Intervirology 6: 168–180.

Summers MD (1971) Electron microscopic observations on granulosis virus entry, uncoating and replication processes during infection of the midgut cells of *Trichoplusia ni.* Journal of Ultrastructural Research 35: 606–625.

Tanada Y & Hess RT (1976) Development of a Nuclear Polyhedrosis Virus in Midgut Cells and Penetration of the Virus into the Hemocoel of the Armyworm, *Pseudaletia unipuncta.* Journal of Invertebrate Pathology 28: 67–76.

Thiem SM & Miller LK (1989) Identification, sequence and transcriptional mapping of the major capsid protein gene of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J Virol 63: 2008–2018.

Tjia ST, Carstens EB & Doerfler W (1979) Infection of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus. II. The viral DNA and the kinetics of its replication. Virology 99: 391–409.

Tomalski MD, Wu J & Miller LK (1988) The location, sequence, transcription, and regulation of a baculovirus DNA polymerase gene. Virology 167: 591–600.

van der Wilk F, van Lent JWM & Vlak JM (1987) Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells. Journal of General Virology 68: 2615–2623.

van Lent JWM, Groenen JTM, Klinge-Roode EC, Rohrmann GF, Zuidema D & Vlak JM (1990) Localization of the 34 kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. Archives of Virology 111: 103–114.

van Oers MM, Flipsen JTM, Reusken CBEM, Sliwinsky EL, Goldbach RW & Vlak JM (1993) Functional domains of the p10 protein of *Autographa californica* nuclear polyhedrosis virus. Journal of General Virology 74: 563–574.

Vaughn JL & Dougherty EM (1985) The replication of baculoviruses. In *Viral Insecticides for Biological Control* pp. 569–633 Edited by K. Maramorosch and KE Sherman, New York: Academic Press.

Vialard JE, Yuen L & Richardson CD (1990) Identification and characterization of a baculovirus occlusion body glycoprotein which resembles spheroidin, an entomopoxvirus protein. Journal of Virology 64: 5804–5811.

Vialard JE & Richardson CD (1993) The 1,629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid- associated phosphoprotein. Journal of Virology 67: 5859–5866.

Vlak JM, Klinkenberg FA, Zaal KJM, Usmany M, Klinge-Roode EC, Geervliet JBF, Roosien J & van Lent JWM (1988) Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-β-galactosidase fusion gene. *Journal of General Virology* 69: 765–776.

Vlak JM, Smith GE & Summers MD (1981) Hybridization selection and *in vitro* translation of *Autographa californica* nuclear polyhedrosis virus mRNA. Journal of Virology. 40: 762–771.

Volkman LE (1983) Occulded and budded *Autographa californica* nuclear polyhedrosis virus: immunological relatedness of structural proteins. Journal of Virology 46: 221–229.

Volkman LE (1986) The 64K envelope protein of budded *Autographa californica* nuclear polyhedrosis virus. Curr Top Microbiol Immunol 131: 103–118.

Volkman LE (1988) *Autographa californica* MNPV nucleocapsid assembly: Inhibition by cytochalasin D. Virology 163: 547–553.

Volkman LE & Zaal KJM (1990) *Autographa californica* M nuclear polyhedrosis virus: Microtubules and replication. Virology 175: 292–302.

Volkman LE, Goldsmith PA, Hess RT & Faulkner P (1984) Neutralization of budded *Autographa californica* NPV by a monoclonal antibody: Identification of the target antigen. Virology 133: 354–362.

Volkman LE & Goldsmith PA (1985) Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: Inhibition of entry by adsorptive endocytosis. Virology 143: 185–195.

Volkman LE, Goldsmith PA & Hess RT (1986) Alternate pathway of entry of budded *Autographa californica* nuclear polyhedrosis virus: fusion at the plasma membrane. Virology 148: 288–297.

Volkman LE & Keddie BA (1990) Nuclear polyhedrosis virus pathogenesis. Seminars in Virology, UC Berkeley 1: 249–256.

Volkman LE & Knudson DL (1986) *In vitro* replication of baculoviruses. In: Granados RR & Federici BA (eds) The biology of baculoviruses. CRC Press, Inc, Boca Raton, pp. 109–127

Volkman LE, Goldsmith PA & Hess RT (1987) Evidence for microfilament involvement in budded *Autographa californica* nuclear polyhedrosis virus production. Virology 156: 32–39.

Volkman LE, Summers MD & Hsieh CH (1976) Occluded and nonoccluded nuclear polyhedrosis virus grown in *Trichoplusia ni*: comparative neutralization comparative infectivity, and *in vitro* growth studies. Journal of Virology 19: 820–832.

Wang X & Kelly DC (1983) Baculovirus replication: Purification and identification of the *Trichoplusia ni* nuclear polyhedrosis virus-induced DNA polymerase. Journal of General Virology 64: 2229–2336.

Wei N & Volkman LE(1992). Hyperexpression of baculovirus polyhedrin and p10 is inversely correlated with actin synthesis. Virology 191:42–48.

Whitford M, Stewart S, Kuzio J & Faulkner P (1989) Identification and sequence analysis of a gene encoding gp67, an abundant envelope glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 63: 1393–1399.

Whitford M & Faulkner P (1992a) A structural polypeptide of the baculovirus *Autographa californica* nuclear polyhedrosis virus contains O-linked *N*-acetylglucosamine. Journal of Virology 66: 3324–3329.

Whitford MF & Faulkner P (1992b) Nucleotide sequence and transcriptional analysis of a gene encodind gp41, a structural glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 66: 4763–4768.

Whitt MA & Manning JE (1988) A phosphorylated 34-kDa protein and a subpopulation of polyhedrin are thiol linked to the carbohydrate layer surrounding a baculovirus occlusion body. Virology 163: 33–42

Wickham TJ, Shuler ML, Hammer DA, Granados RR & Wood HA (1992) Equilibrium and kinetic analysis of *Autographa californica* nuclear polyhedrosis virus attachment to different insect cell lines. Journal of General Virology 73: 3185–3194.

Williams GV, Rohel DZ, Kuzio J & Faulkner P (1989) A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus (AcNPV) p10 gene function using insertion/deletion mutants. Journal of General Virology 70: 187–202.

Wilson M & Miller LK (1986) Changes in the nucleoprotein complexes of a baculovirus DNA during infection. Virology 151: 315–328.

Wilson ME, Mainprize TH, Freisen PD & Miller LK (1987) Location, transcription and sequence of a baculovirus gene encoding a small arginine rich polypeptide. Journal of Virology 61: 661–666.

Wilson ME & Consigli RA (1985a) Characterization of a protein kinase associated with purified capsids of the granulosis virus infecting *Plodia interpunctella.* Virology 143: 516–525.

Wilson ME & Consigli RA (1985b) Functions of a protein kinase activity associated with purified capsids of the granulosis virus infecting *Plodia interpunctella.* Virology 143: 526–535.

Wolgamot GM, Gross CH, Russell RLQ & Rohrmann GF (1993) Immunocytochemical characterization of p24, a baculovirus capsid-associated protein. Journal of General Virology 74: 103–107.

Wood HA (1980) Isolation and replication of an occlusion body deficient mutant of the *Autographa californica* nuclear polyhedrosis virus. Virology 105: 338–344.

Xeros N (1956) The virogenic stroma in nuclear and cytoplasmic polyhedrosis. Nature 178: 412–413.

Yang CL, Stetler DA & Weaver RF (1991) Structural comparison of the *Autographa californica* nuclear polyhedrosis virus-induced RNA polymerase and the three nuclear RNA polymerases from the host, *Spodoptera frugiperda.* Virus Research 20: 251–264.

Young JC, Mackinnon EA & Faulkner P (1993) The architecture of the virogenic stroma in isolated nuclei of *Spodoptera frugiperda* cells *in vitro* infected by *Autographa californica* nuclear polyhedrosis virus. Journal of Structural Biology 110: 141–153.

Zuidema D, Klinge-Roode EC, van Lent JWM & Vlak JM (1989) Construction and analysis of an *Autographa californica* nuclear polyhedrosis virus mutant lacking the polyhedral envelope. Virology 173: 98–108.

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Construction of baculovirus recombinants

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Key words: baculovirus expression system, baculovirus expression vector, recombinant baculovirus

Abbreviations: AcMNPV – *Autographa californica* multinucleocapsid nuclear polyhedrosis virus; SDS – Sodium dodecyl sulphate; PCR – polymerase chain reaction

Introduction

There are three main reasons for wishing to modify baculovirus genomes. The most common arises from the use of baculoviruses to express foreign genes. Baculovirus expression systems frequently give high level expression of proteins with full biological activity. This combination of highly desirable features is responsible for the widespread popularity of baculovirus expression systems. Second, is the renewed interest in the use of baculoviruses as biological insecticides. Wild-type baculoviruses are highly specific insecticides with no toxicity for non-target organisms and have been used to control insect pests for many years (Granados & Federici, 1986). Recently, considerable effort has been made to enhance the insecticidal potency of baculoviruses by introducing foreign genes, such as hormones or toxins, or making other modifications to the viral genome (for reviews see: Wood & Granados, 1991; Bonning & Hammock, 1992). The third reason is that the ability to introduce mutations and other modifications into a baculovirus genome provides a powerful tool for studying baculovirus biology. Studying the complex interactions between baculoviruses and their hosts, both at the cellular and organismal levels, may provide valuable insights into other viral infections. In addition, greater understanding of baculovirus biology is vital for the continued improvement of baculovirus expression systems and baculovirus insecticides.

The large size of baculoviral genomes, 80–200 kb, makes it difficult to manipulate the viral DNA directly due to its sensitivity to shearing and to the paucity of unique restriction sites. The original method for introducing mutations or foreign genes into a baculovirus circumvented these difficulties using a two-step approach; a segment of the viral DNA was cloned into a plasmid vector, modified, and then homologous recombination inside insect cells was used to transfer the modification back into the viral genome (Smith *et al.*, 1983; Pennock *et al.*, 1984). Many recombinant viruses have been made using this method; however, it is inefficient and time consuming. As the need for recombinant viruses grew, more efficient methods were developed. This review will describe the major methods that have been developed to construct recombinant baculoviruses and their relative advantages and weaknesses (for an earlier review see Davies, 1994). The various methods are grouped according to the host in which the recombinant is generated: insect cells, heterologous hosts, and *in vitro.* Most methods for generating recombinants have been developed using AcMNPV, therefore this review will concentrate on this virus.

Generation of recombinant viruses in insect cells

Recombination between a transfer vector and circular viral DNA

The classical method used to introduce a foreign gene or mutation into a baculoviral genome uses an intermediate "transfer vector" (Smith *et al.*, 1983; Pennock *et al.*, 1984). Such vectors consist of a bacterial plasmid into which a segment of the viral DNA has been cloned. This piece of viral DNA can be manipulated by standard molecular biology techniques to create mutations or to introduce foreign genes. Plasmid DNA

from the modified transfer vector is then mixed with circular viral DNA extracted from viral nucleocapsids, and transfected into insect host cells. Inside the cell, host enzymes can mediate recombination between the viral sequences in the transfer vector and the identical sequences in the viral genome. Two recombination events (crossovers), one on either side of the modification, will exchange the modified sequences from the transfer vector for the wild-type viral sequences, generating a recombinant virus (Figure 1, upper panel). However, such double-recombination events occur at a very low frequency and typically only 0.1–1% of the viruses released from the transfected cells are recombinant. The recombination frequency can be boosted two- to three-fold by irradiation of the cells with UV light prior to transfection (Peakman *et al.*, 1989) but this technique is not widely used. The major task is identifying the rare recombinant viruses. In the classical method, wild-type viral DNA is used so that the non-recombinant virus produces plaques that contain the viral occlusion bodies known as polyhedra, whereas recombinant viruses in which a foreign gene has been substituted for the polyhedrin gene give plaques that lack polyhedra. A trained eye can pick out the polyhedra-negative plaques from the background of non-recombinant plaques containing polyhedra. After picking the putative recombinant plaques, three or four rounds of plaque purification are required to eliminate the contaminating wild-type viruses. To obtain a pure recombinant virus by this method takes three to four weeks.

A variant of this approach is to use viral DNA from a virus that expresses β-galactosidase such that plaques of the parental virus stain blue with X-gal, whereas plaques of a recombinant virus are white (Summers & Smith, 1987). Although more appealing to novice users, diffusion of the blue color can obscure the white plaques, and there is a background of non-recombinant white plaques due to the relatively high spontaneous mutation rate of the *lac*Z gene.

Less direct methods have also been employed to identify the recombinant viruses, including: screening by nucleic acid hybridization (Summers & Smith, 1987; Fung *et al.*, 1988; Pen *et al.*, 1989), screening for protein expression using an antibody (Manns & Grosse, 1991; Grosse & Manns, 1995), screening for enzymatic or other activity of the expressed gene, or fluorescence-activated cell sorting (Peng *et al.*, 1993).

The very low frequency of recombination between transfer vectors and circular viral DNA makes screening for recombinant viruses tedious and necessitates several rounds of purification before a pure recombinant virus is obtained. Although this method is still commonly used to construct recombinants from some baculoviruses, for AcMNPV it has largely been superseded by the less time-consuming methods described below.

Use of transfer vectors containing a marker gene cassette

Rare recombinant plaques can be identified easily if they express a marker gene not present in the parental virus. To this end, a cassette consisting of a promoter active in insect cells, the *E. coli lac*Z gene, and a polyadenylation signal has been incorporated into transfer vectors. The marker gene cassette is inserted adjacent to the promoter, cloning sites and polyadenylation signal designed for expression of the target gene, to form a unit that is flanked by viral DNA sequences. One series of such vectors has been constructed by Richardson's group (Vialard *et al.*, 1990; Richardson *et al.*, 1992a; Richardson *et al.*, 1992b, Lalumière & Richardson, 1995) (Invitrogen distributes some of these vectors and their derivatives as pBlueBac vectors), and another series by Vlak's group (Vlak *et al.*, 1990; Zuidema *et al.*, 1990). Recombination of such vectors with viral DNA transfers both expression cassettes to the viral genome resulting in a recombinant virus that expresses β-galactosidase and gives a blue plaque. Although this does not improve the efficiency of recombination, it is much easier to find rare blue plaques amongst a background of white plaques than *vice versa.*

The presence of polyhedra can also be used to identify recombinant plaques if DNA from a virus lacking a functional polyhedrin gene is transfected with a transfer vector containing a polyhedrin expression cassette (Weyer *et al.*, 1990).

The main drawback to using a marker cassette in the transfer vector is that cointegrates, in which the whole transfer vector is inserted into the virus DNA (Figure 1, lower panel), have a similar plaque phenotype to recombinant viruses. Many of the putative recombinant plaques will contain cointegrates because a single crossover integrates the transfer vector into the virus genome (Figure 1, lower panel) whereas two independent crossovers are required to make the desired recombinant (Figure 1, upper panel). Cointegrate viruses are not desirable because they are unstable; recombination between the repeated copies of viral sequences flanking the transfer vector can excise the plasmid and target

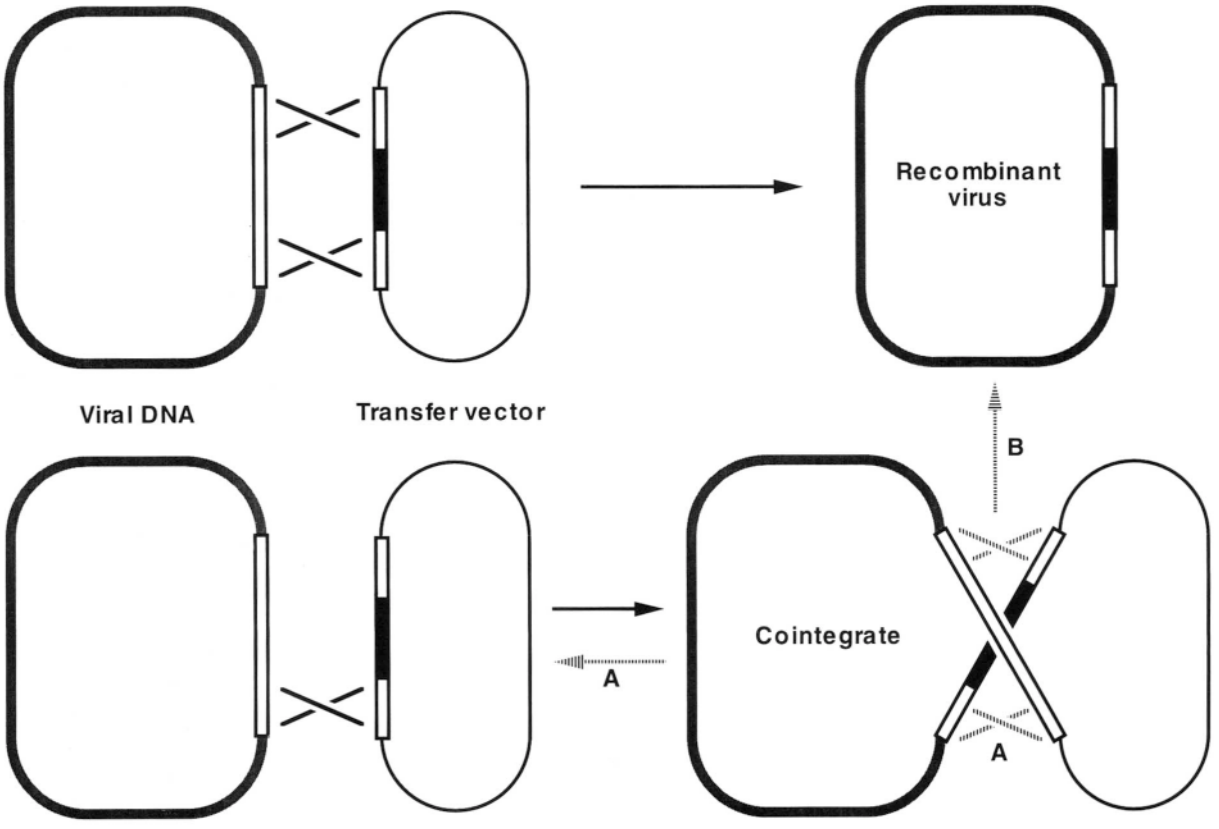

Figure 1. Recombination between circular viral DNA and a transfer vector. Upper panel: Two recombination events, one on either side of the modified sequences, swap a segment of the viral genome for the modified sequences from the transfer vector generating a recombinant virus. Lower panel: A single recombination event integrates the whole transfer vector into the viral genome to generate a large circular DNA called a "cointegrate". A subsequent recombination event can reverse the process (A), or produce the complete recombinant (B).

gene (Figure 1, lower panel) leading to loss of expression. In the classical method, cointegrate viruses have the same plaque phenotype as parental viruses and will be rejected. The isolation of cointegrate viruses when using a transfer vector with a marker cassette can be avoided by transfecting with linear viral DNA (see below). In this case the product of a single crossover is a linear cointegrate which will not be viable.

Other disadvantages of using transfer vectors containing a marker cassette are that the second expression cassette makes the transfer vector large and limits the unique cloning sites, and recombinant viruses express an unwanted protein.

Selection against the parental virus

Godeau *et al.* (1992) constructed viruses that express the thymidine kinase gene from herpes simplex virus type 1 (HSV1-tk) so that Ganciclovir can be used to select against the parental virus. Host cell thymidine kinases do not metabolize the nucleotide analog Ganciclovir whereas HSV1-tk converts Ganciclovir into a toxic inhibitor of DNA replication. In AcMNPVIE–1-tk and AcMNPVIE–1-tk-pl0-SEAP (Godeau *et al.*, 1992), the polyhedrin gene has been replaced by the HSV1-tk gene driven by an immediate early AcMNPV promoter (IE-1(0)). Expression of HSV1-tk makes replication of these viruses sensitive to Ganciclovir. When a standard polyhedrin-based transfer vector is cotransfected with viral DNA from one of these viruses, a double recombination event can replace the HSV1-tk gene with the foreign gene from the transfer vector. Propagation of the progeny viruses in the presence of Ganciclovir selects against the parental viruses and enriches for recombinants. After one cycle of selection, 85% or more of the viruses are recombinant (Godeau *et al.*, 1992).

This method gives a very strong enrichment for recombinant viruses but sacrifices the ability to confirm a virus as recombinant by its plaque phenotype. The major concern when using this method is that mutations in the thymidine kinase gene of the parental virus will give rise to a background of non-recombinant viruses that survive the Ganciclovir selection.

Recombination between a transfer vector and linear viral DNA

Viral DNA that has been linearized at a unique site is 100- to 1000-fold less infectious than circular DNA, but retains the capacity to recombine with transfer vectors that contain homology to the viral DNA on either side of the break (Kitts *et al.*, 1990). By cotransfecting transfer vectors with linearized viral DNA, the background of non-recombinant plaques is greatly reduced but the number of recombinant plaques is affected to a much lesser extent. Consequently, about 25% of the resulting plaques are recombinant (Kitts *et al.*, 1990). The original single-cut virus, AcRP6-SC, has a unique *Bsu*36I site in place of the polyhedrin gene (Kitts *et al.*, 1990), hence it is not possible to visually identify recombinant plaques by the absence of polyhedra. Subsequently, other viruses have been engineered to contain a unique restriction site that allows linearization of the viral DNA but also permits a visual screen for recombinant plaques. Viruses that contain the *E. coli lac*Z gene have a unique *Bsu*36I site that lies within the β-galactosidase coding sequences. By using linearized viral DNA from a *lac*Z virus, plaques of the parental virus are blue and can easily be rejected, raising the proportion of white plaques that are recombinant to 30 to 100% (Kitts *et al.*, 1990, Copeland & Wang, 1993; Kitts, 1995). Two linearizable AcMNPV derivatives that express an intact polyhedrin gene are also available; one, AcV EPA, has a unique *Bsu*36I site following the polyhedrin coding sequences (Hartig & Cardon, 1992), the other has a unique *Sse*8387I site upstream of the polyhedrin gene (Day *et al.*, 1995). Using linear viral DNA from these viruses, plaques of the parental virus contain polyhedra and can be rejected; 50 to 80% of the polyhedra-negative plaques are recombinant (Hartig & Cardon, 1992;Day *et al.*, 1995). Not all plaques with a non-parental phenotype obtained from cotransfections using these linear DNAs are recombinant; the non-parental non-recombinant plaques presumably result from imperfect recircularization of the viral DNA that deletes bases from the junction so that a functional β-galactosidase or polyhedrin gene is no longer expressed (Kitts *et al.*, 1990).

Cotransfections using linear viral DNA give a high proportion of plaques that are recombinant; 10 to 25% without a visual screen, or 30 to 100% when plaques of the parental virus can be rejected because of their phenotype. This makes identifying and purifying recombinant plaques much easier and less time consuming compared with using circular viral DNA. Linear viral DNA can profitably be combined with the use of transfer vectors containing a marker cassette because it eliminates the problem of isolating cointegrate viruses (see above).

The linear viral DNA method has been found to work for either the polyhedrin or p10 loci of AcMNPV, provided a cognate transfer vector is used (Kitts *et al.*, 1990; Kitts, 1995; Martens *et al.*, 1995). AcMNPV viral DNA linearized at either the polyhedrin or p10 locus is commercially available from Invitrogen or PharMingen.

Recombination between linear viral DNA and a transfer vector containing a dominant selectable marker

Lerch & Friesen (1993) investigated the use of the neomycin-resistance gene or the *p*35 gene from AcMNPV as dominant selectable markers and found that the *p*35 gene provided the strongest selection. The *p*35 gene suppresses premature cell death due to induction of apoptosis in Sf21 cells infected with AcMNPV (Clem *et al.*, 1991); consequently production of budded virus from a virus that has a deletion in the *p*35 gene, **vΔ35K,** is greatly reduced. Insertion of a functional *p*35 cassette at another location in the virus restores the production of budded virus to wild-type levels (Hershberger *et al.*, 1992). Thus a transfer vector containing a *p*35 cassette adjacent to the polyhedrin promoter driving expression of the target gene can be used to allow selection of recombinant viruses. Transfection of this vector with **vΔ35K** virus yields recombinants as 30% of the total progeny (Lerch & Friesen, 1993). The proportion of recombinant viruses can be further improved by replacing the **vΔ35K** virus with linearized viral DNA from another virus with a deletion in the *p*35 gene, **vΔ35K/lacZ.** Cotransfection of the *p*35 transfer vector and this linear viral DNA yields 82 to 96% recombinant viruses (Lerch & Friesen, 1993). A pure recombinant virus can be obtained after a single round of plaque purification.

This method gives a very high proportion of recombinant viruses. Its major limitation is that it requires a specialized transfer vector. Another drawback to using this system is that the **vΔ35K/lacZ** virus needed to prepare the linearized viral DNA is produced at a 10-fold lower level than wild-type virus.

Recombination between a transfer vector and linear viral DNA with a "lethal deletion"

A very high proportion of recombinant viruses, approaching 100%, can also be obtained by using a modified linear viral DNA from which essential sequences have been removed such that recombination with a transfer vector is necessary to generate a viable virus (Kitts & Possee, 1993). In AcMNPV the gene downstream of polyhedrin, ORF1629, encodes a phosphoprotein that is associated with the viral nucleocapsid or virion envelope (Vialard & Richardson, 1993; Pham *et al*., 1993) and is essential for production of infectious virus (Possee *et al*., 1991). A *Bsu*36I site was engineered into the C-terminus of ORF1629 without altering the amino acid sequence of the protein, and a second *Bsu*36I site was introduced in ORF603 upstream of the polyhedrin promoter (Kitts & Possee, 1993). The best results were obtained using a virus that also had the *E. coli lac*Z gene in place of the polyhedrin gene and thus contained a third site for *Bsu*36I (Kitts & Possee, 1993). This virus, BacPAK6, is viable; however, restriction of the viral DNA with *Bsu*36I removes a fragment containing part of the essential ORF1629 gene (Figure 2). Recombination between the large fragment of viral DNA and a transfer vector that contains an intact copy of the C-terminus of ORF1629 can rescue the viral DNA by regenerating an intact ORF1629 gene (Figure 2). In this process the foreign gene is also transferred to the viral genome (Figure 2). 90 to 100% of the viruses produced by cotransfecting a transfer vector and BacPAK6 viral DNA digested with *Bsu*36I express the gene from the transfer vector (Kitts & Possee, 1993). This proportion is sufficiently high that it is possible to use the progeny virus stock for protein expression without the need to plaque purify a recombinant virus. Even if a clonal recombinant virus is desired it is easy to isolate a pure recombinant plaque.

The major advantage of this method is the very low background of non-recombinant viruses. A second significant advantage is that it is compatible with a wide variety of transfer vectors that are based on the polyhedrin locus of AcMNPV and hence contain the sequences necessary to rescue restricted BacPAK6 viral DNA. Although the yield of virus from this method is low, it is more than adequate for converting a transfer vector containing a unique insert to a recombinant virus.

BacPAK6 viral DNA digested with *Bsu*36I to remove the ORF1629 C-terminus is commercially available from CLONTECH or as BaculoGold™ from PharMingen.

Generation of recombinant viruses in alternative hosts

To circumvent the low efficiency of recombination inside insect cells, viruses have been modified to contain elements that allow them to replicate as episomes in either yeast or *E. coli.* This allows the viral genome to be manipulated in these more tractable hosts. Once the desired recombinant viral genome has been constructed, DNA can be prepared and transfected into insect cells where the virus will replicate to generate a stock of infectious virus.

Recombination in yeast

Recombination in *Saccharomyces cerevisiae* is highly efficient, especially when one of the participating DNA molecules has a double-strand break, and markers are also available that facilitate selection and screening for recombinant molecules. Patel *et al.* (1992) developed a method to generate recombinant viruses in yeast that takes advantage of these properties. **YCbv::SUP4-o** was constructed by inserting into the polyhedrin locus of AcMNPV the elements required for replication and selection in yeast (Figure 3). When yeast cells are transformed with **YCbv::SUP4-o** DNA, the ARS and CEN elements enable the viral genome to replicate as an episome, and the URA marker can be used to select for cells in which replication of the viral DNA has been established. The SUP4-o ochre suppressing allele of a tRNA gene provides a marker that can either be selected or counterselected. The host strain has an ochre mutation in the ADE2 gene which makes it dependent on adenine for growth and causes the colonies to be pink. A second ochre mutation in the CAN1 gene makes the cells resistant to canavanine. Expression of the SUP4-o gene from **YCbv::SUP4-o** suppresses the ochre mutations of the host so that growth of cells containing this vector is adenine independent but sensitive to canavanine, and the colonies are white. A complementary transfer vector was constructed that contains

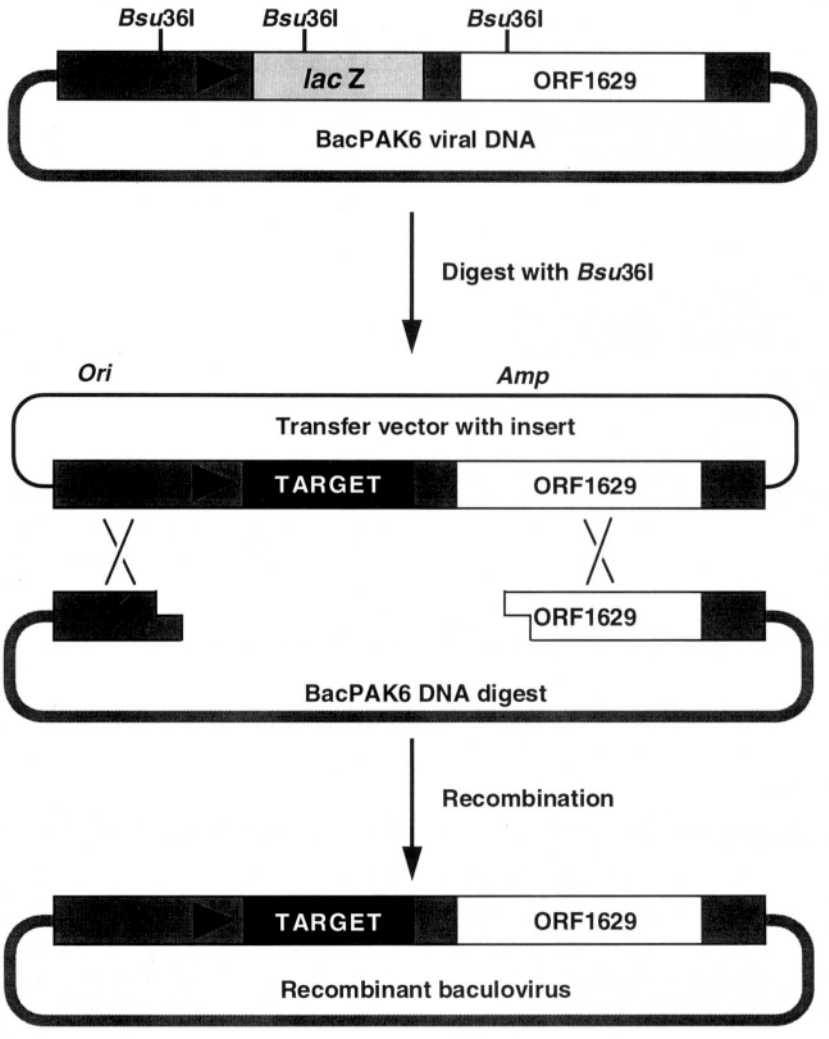

Figure 2. Recombination between a transfer vector and linear viral DNA with a "lethal deletion". Restriction of BacPAK6 viral DNA with *Bsu*36I removes the C-terminus of the essential viral gene, ORF1629. In order to regenerate a viable virus, the large fragment of BacPAK6 must recombine with a transfer vector containing an intact copy of the ORF1629 C-terminus. Recombination restores the integrity of ORF1629 and transfers the target gene to the viral genome.

a cloning site flanked on one side by the AcMNPV polyhedrin promoter and upstream viral sequences, and on the other by the same ARS sequences present in YCbv::SUP4-o. The target gene is cloned into this vector and DNA from the resulting plasmid is digested with restriction enzymes that release a linear DNA fragment carrying the target gene and flanking sequences (Figure 3). If yeast cells containing YCbv::SUP4-o are transformed with the transfer vector digest, homologous recombination can replace the SUP4-o gene in the viral genome with the target gene (Figure 3). In theory, yeast cells in which this has occurred can be selected as pink colonies that are resistant to canavanine; however, a significant background of canavanine resistant mutants necessitates a modified procedure. Yeast cells containing YCbv::SUP4-o are cotransformed with the transfer vector digest and a plasmid containing a TRP gene that complements a mutation making the host dependent on tryptophan. Yeast cells that have taken up DNA are selected on medium lacking tryptophan, and the resulting colonies are screened for canavanine resistance resulting from the loss of the SUP4-o allele. Because SUP4-o makes the cells grow slower, choosing the largest colonies

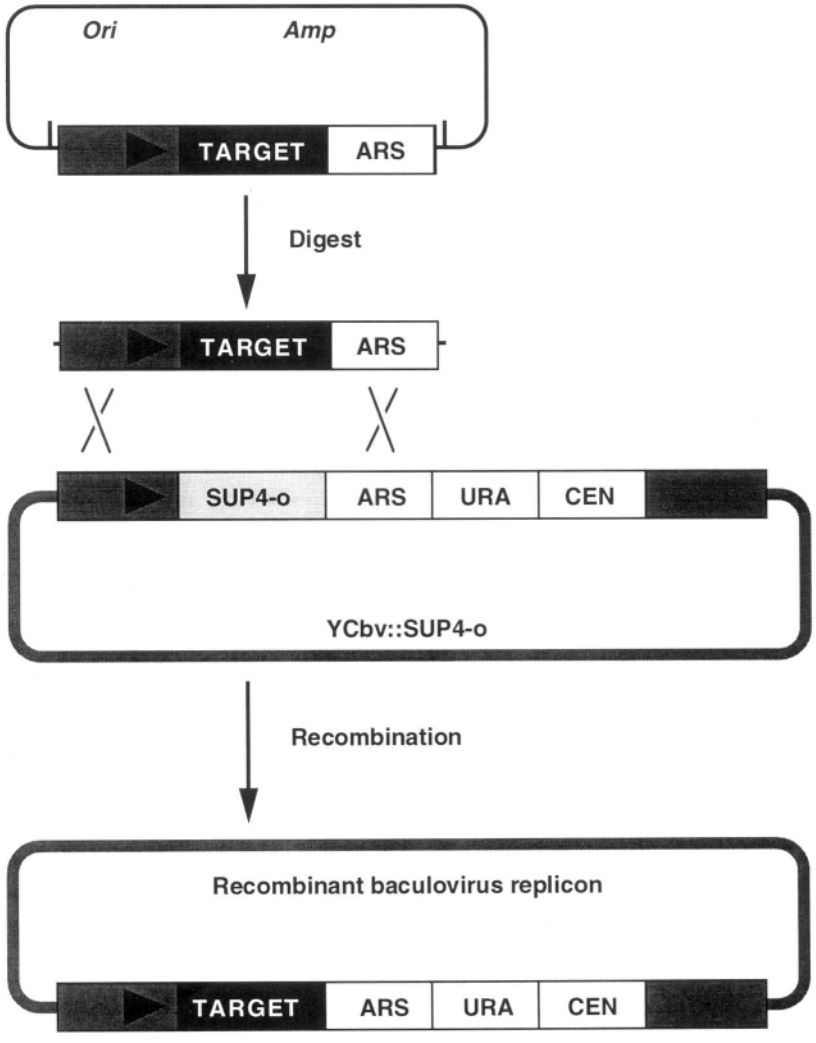

Figure 3. Recombination of a baculovirus genome maintained as an episome in yeast cells.

or pink colonies enriches for the desired clones so that approximately 80% of the selected colonies will be resistant to canavanine (Patel *et al.*, 1992). These colonies harbor a recombinant YCbv vector containing the target gene. After restreaking to obtain a clonal isolate containing a recombinant YCbv, DNA is prepared from the yeast cells , fractionated on a sucrose gradient, and fractions containing viral DNA are located using PCR. Insect cells are then transfected with these fractions, initiating viral replication that generates a stock of recombinant virus.

The advantages of this method are that the recombination frequency is high, identification of colonies containing recombinant virus replicons is relatively easy, and a pure clone can be obtained quickly from a single yeast colony. Limitations of this method are that it requires a specialized transfer vector, and significant labor is required to purify the viral DNA over a sucrose gradient before it can be transfected into insect cells. Although yeast is easy to work with, laboratories not already working with *Saccharomyces cerevisiae* may be deterred by the need to work with another host.

Recombination in Escherichia coli.

Luckow and coworkers have developed a method for generating recombinant baculoviruses in *E. coli* that uses the transposon Tn7 to insert the target gene into the viral genome (Luckow *et al.*, 1993). A baculovirus-plasmid hybrid (bacmid), capable of being propagated

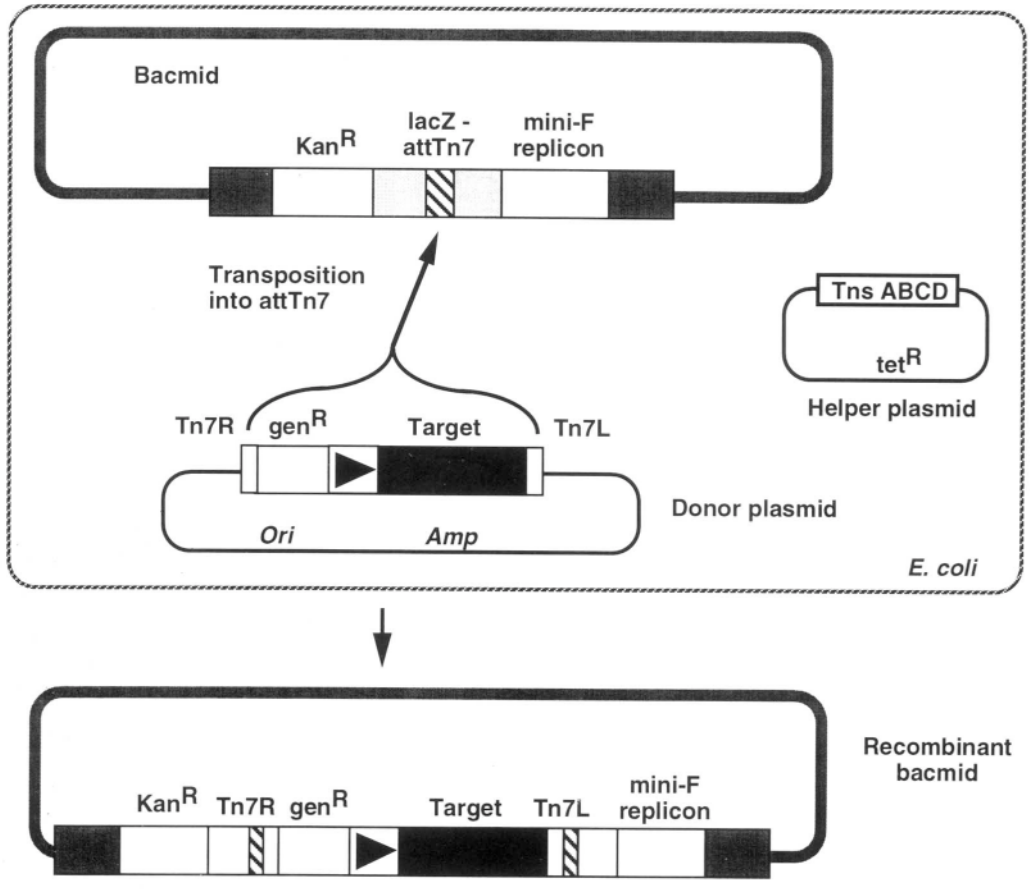

Figure 4. Construction of a recombinant baculovirus by transposition into a bacmid maintained in *E. coli*.

in *E. coli* as a single-copy plasmid was constructed by inserting a minimal replicon derived from the *E. coli* F plasmid and a kanamycin-resistance gene into the polyhedrin locus of AcMNPV (Figure 4). The bacmid also contains the *lacZα* peptide region from a pUC-based plasmid with an in-frame insertion of the target site for Tn7 (att-Tn7). Consequently, colonies of an appropriate host transformed with the bacmid stain blue with X-gal. A specialized donor plasmid carries a mini-Tn7 containing a gentamycin-resistance gene, a copy the AcMNPV polyhedrin promoter followed by a multiple cloning site, and a polyadenylation signal (Figure 4). The target gene is cloned into the donor plasmid downstream from the polyhedrin promoter. To construct a recombinant bacmid, the donor plasmid containing the target gene is transformed into an *E. coli* strain containing both the bacmid and a helper plasmid that expresses the Tn7 proteins necessary for transposition of the mini-Tn7 (Figure 4). Transposition of the mini-Tn7 from the donor plasmid into the att-Tn7 of the bacmid generates a recombinant bacmid (Figure 4). Bacteria harboring the desired product can be identified by screening for colonies which express kanamycin resistance from the bacmid and continue to express gentamycin resistance from the mini-Tn7, but are ampicillin-sensitive because they no longer contain the donor plasmid itself. Approximately half of the **kan^r, gen^r, amp^s** colonies will be white because the mini-Tn7 will have inserted into the att-Tn7 site in the bacmid disrupting the *lacZ* α-peptide. The remaining **kan^r, gen^r, amp^s** colonies will be those in which the mini-Tn7 inserted into the att-Tn7 site in the bacterial chromosome and these will be blue. Typically, 5–25% of all the colonies have the white, **kan^r, gen^r, amp^s** phenotype indicative of a recombinant bacmid (Luckow *et al.*, 1993). After restreaking to obtain a clonal isolate containing a recombinant bacmid, plasmid DNA is prepared and transfected into insect cells.

Once inside insect cells the bacmid replicates as a virus and yields a stock of recombinant baculovirus.

An advantage of this method is that *E. coli* is a familiar host which grows fast and is easy to work with. Once the target gene has been inserted into the donor plasmid, one can quickly generate a recombinant bacmid, obtain a pure clone by restreaking the bacteria, and transfect insect cells to produce a stock of recombinant virus. The method is limited by the requirement for a specialized transfer vector. In addition, distinguishing between colonies containing recombinant and non-recombinant bacmids can be difficult because the bacmid is a single-copy plasmid and generates a much fainter blue color than the familiar multicopy plasmid cloning vectors.

A version of the bacmid system is distributed by Life Technologies, Inc. under the trade name Bac-To-Bac™.

Generation of recombinant viruses *in vitro*

Another way of circumventing the limitations of recombination inside insect cells is to modify the viral DNA *in vitro.* Two different methods for constructing a recombinant virus *in vitro* have been described.

Recombination mediated by Cre

The bacteriophage P1 encodes a recombinase, Cre, that mediates recombination between two copies of a specific site, *lox*P, in the phage genome to circularize the viral DNA after infection and to allow stable inheritance when the viral DNA is replicating as a plasmid (Sternberg & Hoess, 1983). *In vitro*, purified Cre recombinase is sufficient to mediate efficient recombination between any two copies of the 34-bp *lox*P recombination site (Abremski *et al.*, 1983; Hoess & Abremski, 1984). Peakman *et al.* (1992) have made use of the Cre-*lox*P recombination system to generate recombinant baculoviruses *in vitro.* This system uses a derivative of AcMNPV, vAclox, that has a copy of *lox*P in place of the polyhedrin gene (Figure 5). A specialized transfer vector, ploxZ, also contains a *lox*P site and the AcMNPV polyhedrin promoter followed by a multiple cloning site into which target genes are cloned. ploxZ also carries a marker cassette consisting of the AcMNPV p10 promoter, the *E. coli lac*Z gene and a polyadenylation signal (Figure 5). vAclox viral DNA is mixed with plasmid DNA from ploxZ containing the target gene and incubated with Cre *in vitro.* Recombination between the *lox*P site on the plasmid and the *lox*P site in the viral DNA integrates the whole plasmid into the viral genome, generating a recombinant virus (Figure 5). A second round of recombination can reverse the reaction and excise the plasmid. Incubation with Cre therefore generates a mixture of the two input DNAs and recombinant viral DNAs. The recombination products are transfected into insect cells, and the progeny viruses are harvested and plated out to produce individual plaques. Plaques of recombinant virus express β-galactosidase from the marker cassette and stain blue with X-gal. Up to 50% of the viruses produced are recombinant (Peakman *et al.*, 1992). Blue recombinant plaques are picked, purified by repeated plaque assays and then amplified to produce a stock of recombinant virus.

This method very efficiently converts the input viral DNA into recombinant viruses, yielding as many as 5×10^7 recombinants per μg of plasmid DNA (Peakman *et al.*, 1992). Moreover, plaques of recombinant virus are easily identified and may constitute up to 50% of the total plaques. An additional advantage is that no intermediate host is involved. Despite the efficiency with which this system produces recombinant viruses, a plaque assay is still required to identify recombinant plaques and at least one round of plaque purification is required to obtain a clone free of contaminating parental virus. Other disadvantages are that the recombination reaction generates some viruses that have multiple plasmids inserted, and the recombinant viruses express β-galactosidase in addition to the target protein. This method also requires a specialized transfer vector.

Direct cloning into baculovirus DNA

All the methods described above require that the target gene be cloned into a transfer vector as an intermediate step in the construction of a recombinant baculovirus. It is possible to avoid this extra step by directly ligating a DNA fragment into viral DNA that has been linearized at a unique restriction site (Ernst *et al.*, 1994). Viral DNA from Ac-omega, which has a unique site for the intron-encoded endonuclease I-*Sce*I downstream of the polyhedrin promoter, is linearized with I-*Sce*I and dephosphorylated. The target gene is modified so that it is flanked by I-*Sce*I sites that after restriction generate ends compatible with the non-palindromic ends of the linear viral DNA. After ligating the viral DNA and insert for 60 hours, the ligation products are transfected into insect cells, and virus harvested a few days later.

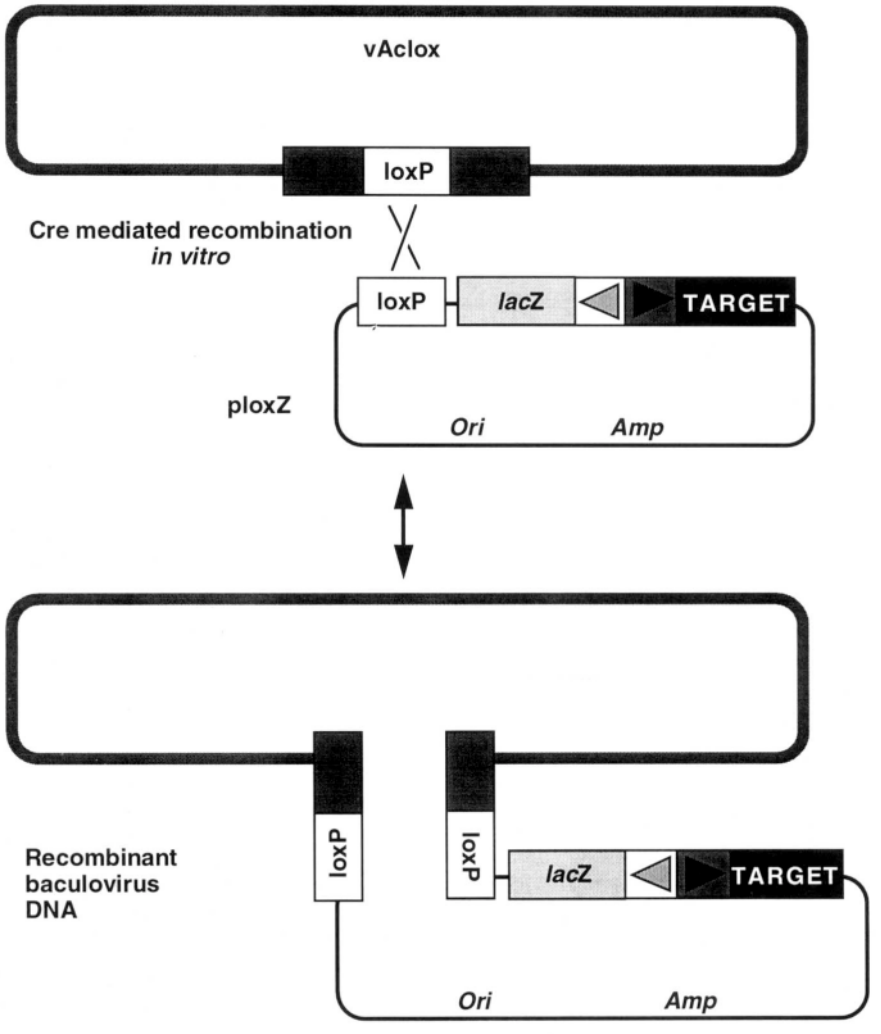

Figure 5. In vitro recombination between transfer vector and viral DNAs mediated by Cre recombinase.

It is possible to obtain more than 6×10^5 recombinant viruses per mg of viral DNA, with the non-recombinant background estimated to be less than 5% (Ernst *et al.*, 1994).

Although simple, this direct approach is limited to the use of a few endonucleases, *Bsu*36I, *Srf*I, *Sse*8387I and I-*Sce*I, that do not cut the wild-type AcMNPV genome (Ayres *et al.*, 1994). Furthermore it lacks the experimental flexibility provided by a transfer vector. However, direct cloning into the viral DNA may be suitable for constructing libraries of recombinant baculoviruses.

Appropriate methods for particular applications

Expression of foreign genes

Several of the methods described above can be used to generate one or more individual recombinant viruses quickly and efficiently (Table 1). Many users will find the convenience of a commercially available system appealing since this avoids the labor involved in preparing high-quality linear viral DNA. On the other hand, the bacmid and yeast systems use a host strain carrying the baculovirus as an episome and avoid the need for viral DNA altogether.

Another important factor to consider is the availability of compatible transfer vectors providing options

Table 1. Comparison of modern methods for constructing recombinant baculoviruses

Method	Available commercially	Variety of compatible transfer vectors	Suitability for:		
			Foreign gene expression	Introduction of potentially lethal mutations	Expression library construction
Ganciclovir counter-selection	No	Yes	Good	None	Poor
Linear viral DNA + transfer vector with marker cassette	Yes	Yes	Good	None	Very poor
Linear viral DNA + transfer vector with dominant selectable marker	No	No	Very good	None	Poor
Linear viral DNA with lethal deletion	Yes	Yes	Very good	None	Poor
Recombination in yeast	No	No	Very good	Very good	Very poor
Bacmid	Yes	No	Very good	Very good	O.K.
Site-specific recombination *in vitro*	No	No	O.K.	None	Poor
Direct cloning	No	Not applicable	O.K.	None	Good

such as secretion signals, tags for affinity purification, multiple promoters, or alternative promoters (for review see López-Ferber *et al.*, 1995). For convenience and versatility it is hard to beat the use of linearized viral DNA with a lethal deletion. The methods that require a specialized transfer vector are currently limited to vectors with the standard polyhedrin promoter without any fusion tags or secretion signals, although modified versions could easily be constructed.

The methods that use *E. coli* or yeast as an alternative host and yield a pure clone of recombinant virus without any plaque assays may appeal to the novice user who frequently has difficulty obtaining good plaques. However, plaque assays are generally required later to get the accurate virus titer needed to optimize expression of the recombinant protein.

Ultimately, the decision over which system to use will be based on personal preference, experience, and the availability of local expertise.

Introduction of potentially lethal mutations

In the study of baculovirus biology, it is frequently desirable to knock out a viral gene in order to understand its function. If the target gene is essential, it will be impossible to construct such a mutation using methods that rely on virus replication inside insect cells. The only methods that allow the introduction of potentially lethal mutations are those that use an alternative host, either *E. coli* or yeast, to propagate the recombinant viral genome (Table 1).

Expression library construction

For some purposes it may be desirable to convert a mixed population of target genes, e.g. a pool of mutants or a cDNA population, into a library of recombinant baculoviruses. This requires a method that (i) efficiently converts the input population of fragments or transfer vectors into recombinant virus, and (ii) produces progeny viruses with a sufficiently high proportion of recombinants that the pool of virus can be used without any further selection. None of the existing methods are ideally suited for this purpose (Table 1). Linear viral DNA with a lethal deletion, and linear viral DNA with a transfer vector using *p*35 as a dominant selectable marker, produce close to 100% recombinants; however, the low efficiency with which transfer vector is converted to virus will preclude the use of these methods for complex pools. Conversely, *in vitro* site-specific recombination is very efficient at converting the input plasmid to virus, but at best yields only 50% recombinants. The bacmid system should consistently give about 50% recombinants with good efficiency hence this method may be acceptable for generating libraries. Direct cloning into linearized baculovirus DNA is analogous to the construction of libraries in phage or plasmid vectors and is probably the existing method most suitable for constructing libraries.

Production of libraries of very high complexity will require further advances in baculovirus recombinant DNA technology. For instance, it may be possible to achieve efficient conversion of plasmid into virus and a high yield of recombinants by combining the *in vitro* site-specific recombination system with the Ganciclovir, *p35*, or ORF1629 selections for recombinant viruses.

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References

Abremski K, Hoess R & Sternberg N (1983) Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. Cell 32: 1301–1311.

Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M & Possee RD (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202: 586–605.

Bonning BC & Hammock BD (1992) Development and potential of genetically engineered viral insecticides. In: Tombs MP(ed.) Biotechnology and genetic engineering reviews, Vol. 10(pp.455–489) Intercept, Andover.

Clem RJ, Fechheimer M & Miller LK (1991) Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science 254: 1388–1390.

Copeland WC & Wang TS-F (1993) Mutational analysis of the human DNA polymerase a. J. Biol. Chem. 268: 11028–11040.

Day A, Wright T, Sewall A, Price-Laface M, Srivastava N & Finlayson M (1995) Rapid procedures for the isolation and PCR analysis of recombinant baculovirus. In: Richardson (ed.) Methods in Molecular Biology, Vol. 39: Baculovirus Expression Protocols (pp. 143–159) Humana Press, Totowa, NJ.

Davies AH (1994) Current methods for manipulating baculoviruses. Bio/Technology 12: 47–50.

Ernst WJ, Grabherr RM & Katinger HWD (1994) Direct cloning into the *Autographa californica* nuclear polyhedrosis virus for generation of recombinant baculoviruses. Nucleic Acids Res. 22: 2855–2856.

Fung M-C, Chiu KYM, Weber T, Chang T-W & Chang NT (1988) Detection and purification of a recombinant human B lymphotropic virus hhv–6 in the baculovirus expression system by limiting dilution and DNA dot-blot hybridization. J. Virol. Meth. 19: 33–42.

Godeau F, Saucier C & Kourilsky P (1992) Replication inhibition by nucleoside analogues of a recombinant *Autographa californica* multicapsid nuclear polyhedrosis virus harboring the herpes thymidine kinase gene driven by the IE–1(0) promoter: a new way to select recombinant baculoviruses. Nucleic Acids Res. 20: 6239–6246.

Granados RR & Federici BA (1986) The biology of baculoviruses, Vol. II. CRC Press, Boca Raton, Florida.

Grosse F & Manns A (1995) Fast isolation of recombinant baculovirus by antibody screening. In: Richardson (ed.) Methods in Molecular Biology, Vol. 39: Baculovirus Expression Protocols (pp. 179–185) Humana Press, Totowa, NJ.

Hartig PC & Cardon MC (1992) Rapid efficient production of baculovirus expression vectors. J. Virological Methods 38: 61–70.

Hershberger PA, Dickson JA & Friesen PD (1992) Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. J. Virol. 66: 5525–5533.

Hoess R & Abremski K (1984) Interaction of the bacteriophage P1 recombinase Cre with the recombining site *lox*P. Proc. Natl. Acad. Sci. USA 81: 1026–1029.

Kitts PA (1995) Production of recombinant baculoviruses using linearized viral DNA. In: Richardson (ed.) Methods in Molecular Biology, Vol. 39: Baculovirus Expression Protocols (pp. 129–142) Humana Press, Totowa, NJ.

Kitts PA, Ayres MD & Possee RD (1990) Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res. 18: 5667–5672.

Kitts PA & Possee RD (1993) A method for producing recombinant baculovirus expression vectors at high frequency. BioTechniques 14:810–817.

Lalumière M & Richardson CD (1995) Production of recombinant baculoviruses using rapid screening vectors that contain the gene for β-galactosidase. In: Richardson (ed.) Methods in Molecular Biology, Vol. 39: Baculovirus Expression Protocols (pp. 161-177) Humana Press, Totowa, NJ.

Lerch RA & Friesen PD (1993) The 35-kilodalton protein gene (*p*35) of *Autographa californica* nuclear polyhedrosis virus and the neomycin resistance gene provide dominant selection of recombinant baculoviruses. Nucleic Acids Res. 21: 1753–1760.

López-Ferber M, Sisk WP & Possee RD (1995) Baculovirus transfer vectors. In: Richardson (ed.) Methods in Molecular Biology, Vol. 39: Baculovirus Expression Protocols (pp. 25–63) Humana Press, Totowa, NJ.

Luckow VA, Lee SC, Barry GF & Olins PO (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli. J.* Virol. 67: 4566–4579.

Manns A & Grosse F (1991) Fast isolation of recombinant baculovirus by antibody screening. BioTechniques 10: 154–158.

Martens JWM, van Oers MM, van de Bilt BD, Oudshoorn P & Vlak JM (1995) Development of a baculovirus vector that facilitates the generation of pl0-based recombinants. J. Virological Meths. 52: 15–19.

Patel G, Nasmyth K & Jones N (1992) A new method for the isolation of recombinant baculovirus. Nucleic Acids Res. 20: 97–104.

Peakman T, Page M & Gewert D (1989) Increased recombinational efficiency in insect cells irradiated with short wavelength ultraviolet light. Nucleic Acids Res. 17: 5403.

Peakman TC, Harris RA & Gewert DR (1992) Highly efficient generation of recombinant baculoviruses by enzymatically mediated site-specific *in vitro* recombination. Nucleic Acids Res. 20: 495–500.

Pen J, Welling GW & Welling-Wester S (1989) An efficient procedure for the isolation of recombinant baculovirus. Nucleic Acids Res. 17:451.

Peng S, Sommerfelt MA, Berta G, Berry AK, Kirk KL, Hunter E & Sorscher EJ (1993) Rapid purification of recombinant baculovirus using fluorescence-activated cell sorting. BioTechniques 14: 274–277.

Pennock GD, Shoemaker C & Miller LK (1984) Strong and regulated expression of *Escherichia coli* β-galactosidase in insect cells with a baculovirus vector. Mol. Cell. Biol. 4: 399–406.

Pham DQ-D, Hice RH, Sivasubramanian & Federici BA (1993) The 1629-bp open reading frame of the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus encodes a virion structural protein. Gene 137: 275–280.

Possee RD, Sun T-P, Howard SC, Ayres MD, Hill-Perkins M & Gearing KL (1991) Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis 9.4 kbp *Eco*RI-I and -R (polyhedrin gene) region. Virology 185: 229–241.

Richardson C, Attia J, Dunn R, Gupta S, O'Connor M, Semeniuk D, Tam J, Hamel M, Lambert G, Dennis M, Jacobs F, Martin L, Iorio C & Vialard J (1992a) Engineering glycoproteins for secretion using the baculovirus expression system. In: Vlak JM, Schlaeger E-J & Bernard AR (eds) Baculoviruses and recombinant protein production processes (pp. 67–74) Roche, Basel.

Richardson CD, Banville M, Lalumière M, Vialard J & Meighen EA (1992b) Bacterial luciferase produced with rapid screening baculovirus vectors is a sensitive reporter for infection of insect cells and larvae. Intervirology 34: 213–227.

Smith GE, Fraser MJ & Summers MD (1983) Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. J. Virol. 46: 584–593.

Sternberg N & Hoess R (1983) The molecular genetics of bacteriophage P1. Ann. Rev. Genet. 17: 123–154.

Summers MD & Smith GE (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin No. 1555.

Vialard J, Lalumière M, Vernet T, Briedis D, Alkhatib G, Henning D, Levin D & Richardson C (1990) Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the β-galactosidase gene. J. Virol. 64: 37–50.

Vialard JE & Richardson CD (1993) The 1,629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. J. Virol. 67: 5859–5866.

Vlak JM, Schouten A, Usmany M, Belsham GJ, Klinge-Roode EC, Maule AJ, van Lent JWM & Zuidema D (1990) Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. Virology 179: 312-320.

Weyer U, Knight S & Possee RD (1990) Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors. J. Gen. Virol. 71: 1525–1534.

Wood HA & Granados RR (1991) Genetically engineered baculoviruses as agents for pest control. Ann. Rev. Microbiol. 45: 69–87.

Zuidema D, Schouten A, Usmany M, Maule AJ, Belsham GJ, Roosien J, Klinge-Roode EC, van Lent JWM & Vlak JM (1990) Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. J. gen. Virol. 71:2201–2209.

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Passage effect of virus infection in insect cells

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Introduction

Genomic plasticity is the hallmark of all biological processes and for viruses is more apparent because of their short generation times and high number of "progeny" produced during each replication cycle or passage. Despite the inherent fidelity of DNA replication, DNA genomes, including those of baculoviruses, continuously undergo variations in their nucleotide sequences. Due to recombination and errors in DNA replication there is a certain probability of error at each passage which for the baculovirus genome leads to point mutations, deletions, rearrangements, inversions, reiterations and acquisition of host cell DNA. It is in this background that baculoviruses replicate either in cells of insect larvae or of insect tissue cultures. Whether the baculovirus replicates in its insect host as a "natural" pathogen, or in cultured cells for the production of microbial insecticides and foreign proteins of economic or scientific interest, the potential for such genomic alterations is omnipresent.

Faulkner (1981) first suggested that the production of variants, atypical morphogenesis and lower virulence of baculovirus preparations occurred as a consequence of repeated serial passage, herein referred to as the "passage effect". The result of prolonged (undiluted) passage of baculovirus *in vitro*, was first shown by MacKinnon *et al.* (1974) for *Trichoplusia ni* NPV (TnNPV) derived from "late passage" (up to 50) in *T. ni* TN–368 cells. At late passage fewer polyhedra per cell and less virulent polyhedra were produced. Moreover polyhedral morphology mutants and aberrant and shorter nucleocapsids resulted and the number of normal occluded virions per polyhedral inclusion body (PIB; the form of baculoviruses responsible for insect to insect horizontal transmission) also decreased. Payne (1988) noted the importance of passage effect in the production of effective viral pathogens for insect control. Tramper & Vlak (1986) first recognized the importance of the passage effect in bioreactor production of baculoviruses and foreign protein in the context of decreased productivity.

The accumulation of genomic alterations through several replication cycles either through individual insects or tissue culture flasks or through the several cycles of replication in bioreactors can be referred to as the passage effect. Some of these new genotypic variants could replicate at the expense of the original parental virus and could, with sufficient numbers of passages become dominant. The major problems with the passage effect on baculovirus replication and expression is the loss of virulence of PIB for virus grown in tissue culture and, for viruses used as expression vectors, a reduced ability to produce the foreign protein of interest. For growth of PIB in tissue culture there is no selection pressure for virus capable of production of infectious polyhedra. Similarly for foreign genes in recombinant baculoviruses there is often no selection to maintain and express those genes.

Baculovirus genomic alterations

That genomic alterations occur frequently among baculoviruses was evident from the many genotypic variants (McIntosh *et al.*, 1987) initially detected by restriction fragment length polymorphism of many natural baculovirus isolates including *Autographa californica* nuclear polyhedrosis virus (AcMNPV; Lee & Miller, 1978; Miller, 1984), *Spodoptera frugiperda*

NPV (SfNPV; Knell & Summers, 1981), *S. exempta* MNPV (Brown *et al*., 1985), *S. litura* NPV (Maeda *et al*., 1990) and *Panolis flammea* MNPV (Weitzman *et al*., 1992). That baculovirus genomes can recombine was initially demonstrated by Summers *et al.* (1980) for AcMNPV and *Rachiplusia ou* NPV and also by recombination between mutant strains of AcMNPV (Carstens *et al*., 1987). Moreover the development of baculoviruses as expression vectors is predicated on homologous recombination between baculovirus and transfer vector DNAs.

Of the many genomic changes that can occur some are silent and provide no selective advantage, some are lethal and, except under unusual circumstances, would be eliminated with passage. Certain changes could enhance replicative advantage so that the mutant virus could outcompete the original genotype during successive passages. A single nucleotide change which alters a codon but not the corresponding amino acid could, except for factors such as codon usage (Ayres *et al*., 1994), have no effect. An alteration in a codon leading to substitution of a different amino acid could lead to proteins such as polyhedrin with altered structure (e.g., mutant M29 and M934, Carstens *et al*., 1987; 1992) or influence the activity of viral enzymes critical for viral replication such as the AcMNPV helicase (Lu & Carstens, 1991). Other changes such as deletions or insertions of DNA in an essential gene, could alter the reading frame. If deletions are large enough, entire genes could be eliminated. Another alteration which could influence gene expression is reiteration of viral DNA such as reported by Lu & Carstens (1990). Those genotypic changes which enhance the replication ability in host cells, or insects will be amplified at each passage and will eventually dominate the original genotype. The outcome depends on the virus species and genotype, the cell (species and tissue source of cells in culture or in insects), the nature of the genotypic changes (genes affected, source and location of DNA insertions or deletions), the method of virus culture (passage in insects, cells in suspension, monolayers or bioreactors, frequency of transfer) and multiplicity of infection (moi).

For AcMNPV and other baculoviruses there are numerous isolates, strains, variants or mutants which have been extensively analyzed to determine the nature of the genotypic variations (Eraser, 1987; Kool *et al*., 1991; Kool&Vlak, 1993; Xiong *et al*., 1991; Table 1). Genotypic variation in the AcMNPV and other baculovirus genomes, include point mutations, both small and large deletions and insertions, recombination and reiterations. There may be hot spots for certain genomic alterations such as insertions due to transposable elements or the deletions in the hypervariable DA26 gene region (O'Reilly *et al*., 1990), but all regions of the genome appear capable of variation. Some variations such as transposon-mediated insertions occur readily even under low passage number and low moi (Fraser *et al*., 1985). Other variations occur under conditions of genomic stress during prolonged passage (Kumar & Miller, 1987; O'Reilly & Miller, 1990; Miller & Miller, 1982; Friesen *et al*., 1986) or under high multiplicity passage (HMP) in flasks or bioreactors (Kool *et al*., 1991).

Deletions of viral DNA occurs readily. For example, the genomes of the majority of the 667 AcMNPVLl-X plaques analyzed by Kumar & Miller (1987), after only 10 passages at an moi of 0.1, had deletions of 0.7 kb in *Pst*1 G (335) or 0.05 kb in *Pst*1 I (321). Many other deletions ranging in size from 0.1 to 2.3 kb have been reported and some are summarized in Table 1. Some deletions inactivate viral genes such as the UDPecdysteroid glucosyltransferase gene (*egt*; O'Reilly & Miller, 1990) and the DA26 gene (O'Reilly *et al*., 1990). Since these mutants survive, neither gene is essential for replication in cell culture. The DA26 deletion occurred after passage of AcMNPV through *Manduca sexta* larvae suggesting that deletions can also occur by passage in insects. One consequence of the passage effect is that some of the genotypic changes which arise through passage involve sequential and cumulative deletions of viral DNA (Kool *et al*., 1991; Cusack & McCarthy, 1989; Wickham *et al*., 1991; Lee & Krell, 1992; 1994; Croizier *et al*., 1985; Figure 1) resulting in major losses of the standard genome. Such highly deleted genomes are released from the infected cells as particles referred to as defective interfering particles (DIP; Huang & Baltimore, 1977). A deletion of 55 kb occurs during replication of M5, an AcMNPV PIB morphology mutant (Carstens, 1982; 1987) and in the generation of AcMNPV DIP in a bioreactor (Kool *et al*., 1991). Since DIP lack many essential genes they cannot independently infect cells. However, at HMP both DIP and helper virus can co-infect the same cell allowing the DIP genome to replicate and be expressed at the expense of the helper virus (Roux *et al*., 1991; Bangham & Kirkwood, 1990). Moreover the DIP concentration increases relative to the original parental strain (wild type virus or recombinant virus) and the virus titre and foreign gene expression would decline with each passage (MacKinnon *et al*., 1974; De Grooijer *et al*., 1992; Kompier *et al*., 1988; van Lier *et*

Table 1. Location of DNA insertions (▼) deletions (▲) or reiterations (▼ n) in AcMNPV DNA

#	Map units	▼ or ▼, size	Virus mutant, strain, description, fragment, cells	Reference
1	0.3–5.8	▼ 1.5 kb	4a20 (AcMNPV L1-X; *Pst*1 E)	Kumar & Miller, 1987
2	1.9–44.2	▲ 55.6 kb	AcMNPV E2, start of deletion in DI	Kool *et al.*, 1991
3	2.8	▼ 290 bp	M5, HR–3, transposon termini from *S. frugiperda*	Carstens, 1987
4	3.5	▼ 3117 bp	AdE1–192 (AcMNPV E; *Eco*R1 H)	Xiong *et al.*, 1991
		▼ variable	in many recombinant baculoviruses	Luckow, 1991
		point	M5, AcMNPV HR–3, loss of *Bam*H1 site	Carstens, 1987
		point	M29, AcMNPV HR–3, loss of *Hin*dIII site	Duncan, 1983; Carstens *et al.*, 1987
5	7.2–12.9	▲ 0.7 kb	1a7 (AcMNPV L1-X; 335/667 plaques; *Pst*1 G)	Kumar & Miller, 1987
6	8.5–10.1	▲ 2141 bp	vMSLB, (AcMNPV L1, DA26, *M. sexta* larvae)	O'Reilly *et al.*, 1990
7	8.9–9.7	▲ 1085 bp	1a7 (AcNPV L1-X; *egt*-; FP)	Kumar & Miller, 1987
				O'Reilly & Miller, 1990
8	9.2–10.0	▲ 1288 bp	vMSLA (AcMNPV L1, DA26, *M. sexta* larvae)	O'Reilly *et al.*, 1990
9	10.0	▼ 1530 bp	4c19 (AcNPV L1-X; FP, 10 passages in TN–368)	Kumar & Miller, 1987
				O'Reilly *et al.*, 1990
10	10.2	▼ 780 bp	5a25 (AcNPV L1-X; FP, 10 passages in TN–368)	Kumar & Miller, 1987
				O'Reilly *et al.*, 1990
11	14.0–18.0	▼ 0.05 kb	4c24 (AcNPV L1-X; FP, *Pst*1 I, FP)	Kumar & Miller, 1987
		▼ 1.1 kb	ldl (AcNPV L1-X; FP, *Pst*1 I, FP)	
		▲ 0.05 kb	2d23 plus (AcNPV L1-X; 321/667 plaques, FP)	
12	≈20	▼ 11.2 kb	AcMNPV E2, reiterated baculovirus sequences	Burand & Summers, 1982
13	20.1–23.3	▼ 1.1 kb	2b11 (AcNPV L1-X; *Pst*1 K)	Kumar & Miller, 1987
		▼ 2.5 kb	2d23 (AcNPV L1-X; *Pst*1 K)	
14	29.0–30.0	▼ 1.05 kb	6a18 (AcNPV L1-X; *Eco*R1 T)	Kumar & Miller, 1987
15	32.2–37.2	▲ 2.3 kb	AcFP875–1 (AcMNPV E2)	Beames & Summers, 1988
16	34	▼ 2581 bp	Ad2E1-D (AcMNPV E, *Eco*R1 O)	Xiong *et al.*, 1991
17	34.7–37.1	▼ 0.4–1.1 kb	5d3, 0.4 kb; 5c25, 0.8 kb; 5a11, 1.1 kb (*Pst*1 E)	Kumar & Miller, 1987
18	36.7–36.8	▲ 0.1 kb	AcFP480–1 (AcMNPV E2)	Beames & Summers, 1988
19	36.8	▼ 1.2 kb	AcFP1 (AcMNPV E2, *T. ni* DNA; TN–368)	Fraser *et al.*, 1983
		▼ 2164 bp	AcFP480–2 (IFP2.2; *S. frugiperda* DNA)	Beames & Summers, 1988; 1990
20	36.9	▼ 830 bp	AcFP6 (AcMNPV E2, *T. ni* transposon TFP3/2)	Beames & Summers, 1988; 1990
		▼ 1565 bp	AcFP875–2 (AcMNPV E2, IFP1.6; *S. frugiperda*)	Cary *et al.*, 1989
		▼ 2.5 kb	AcFP3/AcFP4 (AcMNPV E2 *T. ni* DNA TN–368)	Fraser *et al.*, 1983
		▼ 2.8 kb	AcFP5 (AcNPV E2, IFP2; *T. ni* DNA, TN–368)	Wang *et al.*, 1989
21	42.8–52.5	▼ ≈3 kb	Ad2E1-B (AcMPNPV E; *Eco*R1 C)	Xiong *et al.*, 1991
22	45.1	▼ 290 bp	M5 (AcMNPV H6R–3 *S. frugiperda* transposon)	Carstens, 1987
23	68.3–69.8	▼ 0.5 kb	3d4 (AcNPV L1-X; *Eco*R1 Q)	Kumar & Miller, 1987
24	≈69	▼ 5.85 kb	spB5 (MP, AcMNPV E2, 30 passages, TN–368)	Burand & Summers, 1982
		▼ 6.12 kb	spB6 (MP, AcMNPV E2, 30 passages, TN–368)	
		▼ 6.12 kb	spC6 (MP, AcMNPV E2, 30 passages, TN–368)	
25	69.9–72.7	▼ ≈3 kb	Ad2E1-A/E (AcNPV E, intermed. FP, *Eco*R1 L)	Xiong *et al.*, 1991
26	76.1–78.3	▼ 0.08 kb	3b29 (AcNPV L1-X; *Pst*1 L)	Kumar & Miller, 1987
27	82.3	▼ 634 bp	transposon	Gombart *et al.*, 1989
28	86.2	▼ 0.27 kb	FP-DS, (AcMNPV L1, LTR of TED only)	Miller & Miller, 1982
		▼ 7.3 kb	FP-DL, (AcMNPV L1, LTR and TED DNA)	Friesen *et al.*, 1986
			(both *Hin*dIII K, 25 passages in TN–368)	Friesen & Nissen, 1990
		▼ n 2.8 kb	defective genomes (AcMNPV E2)	Lee & Krell, 1992; 1994
29	88.3–89.9	▼ 0.5 kb	1c4 (AcNPV L1-X; intermediate FP)	Kumar & Miller, 1987
		▼ ≈3 kb	Ad2E1-C (AcMNPV E, *Eco*R1 Q)	Xiong *et al.*, 1991
30	90.3	▼ n 1.4 kb	AcMNPV HR–3, ts8A reiterations (*Eco*R1 P)	Carstens & Lu, 1990

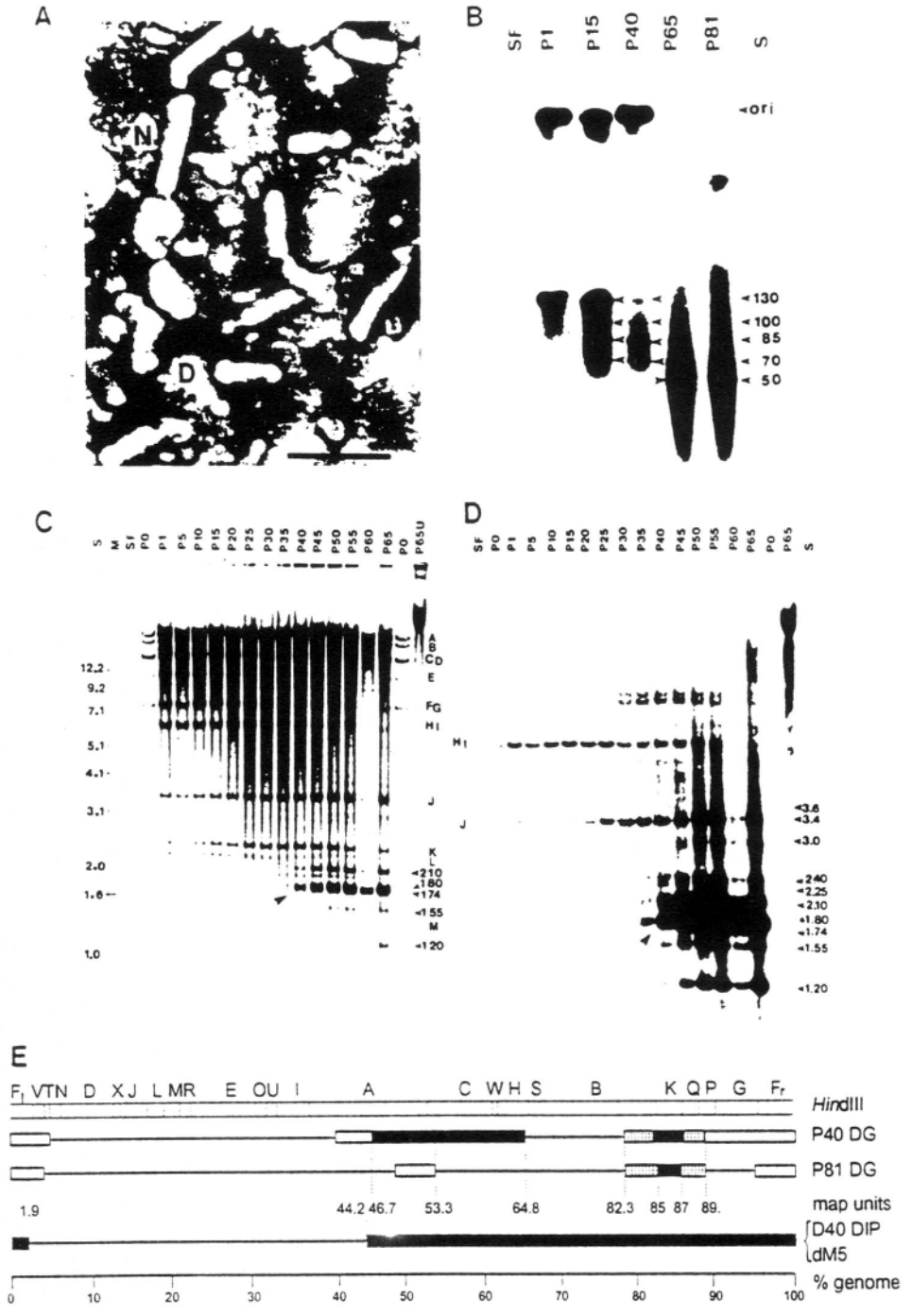


Figure 1. Generation of DIP and defective genomes by high multiplicity passage of AcMNPV. A) Electron microscopy of normal (N) and shorter, defective interfering particles (D) taken at day 40 from a bioreactor. The bar represents 300 nm. B) Pulsed field gel electrophoresis of intracellular AcMNPV defective genomes at passage numbers P1 to P81 as indicated. Arrowheads, with sizes (kb) indicated to the right, point to some of the DNA bands in P15 to P81. C and D) Southern blot analysis of *Xho*1 digested intracellular defective genomes at passage numbers indicated above each lane (P0-P65) or from uninfected cells (lane SF); C was probed with standard virus DNA and D was probed with gel-purified (30–100 kb) defective genome DNA. Arrowheads point to the hypermolar *Xho*1 DNA fragments at 1.8 kb. E) Summary of mapping of deletions on the *Hin*dIII map (*Hin*dIII) in defective genomes at passage 40 (P40 DG) and 81 (P81 DG), DIP at day 40 from a bioreactor (D40 DIP) and the deleted genome in M5 preparations (dM5). Single lines indicate deleted regions of the genome and the degree of shading in the bars in P40 DG and P81 DG indicates the relative proportion of those fragments in those defective genome preparations. A is from Kool *et al.*, 1991; B and D and data from E are from Lee & Krell, 1992 and Kool & Vlak, 1993.

al., 1992). DIP can also mediate persistent infections as observed for *Heliothis zea* NPV (Hz–1) DIP in IMC Hz–1 and *T. ni* TN–368 cells (Wood & Burand, 1986).

Insertions of DNA into baculovirus genomes can also occur at high frequency. Kumar & Miller (1987) found 100 of 667 viral plaques with insertions of 0.08 to 2.5 kb of DNA, the majority of which (88) were in *Pst*1 E. The sizes of other insertions in baculovirus genomes range from 0.3 to 3.1 kb and up to 11.2 (Table 1) in both standard and recombinant baculoviruses (Luckow, 1991). Insertion of DNA is usually mediated by homologous recombination, as for the generation of recombinant baculoviruses (Luckow, 1991) and as demonstrated by the efficiency of marker rescue (Carstens *et al*., 1987). However DNA insertions can also be mediated by non-homologous recombination as shown by Xiong *et al*. (1991) using adenovirus DNA and, as is becoming increasingly obvious, by transposons (Miller & Miller, 1982; Fraser *et al*., 1983, 1985; Beames & Summers, 1990; Gombart *et al*., 1989; Cary *et al*., 1989; Wang *et al*., 1989; O'Reilly *et al*., 1990; Blissard & Rohrmann, 1990). DNA insertions can be from the cell (e.g., transposons), co-infecting viruses (Summers *et al*., 1980; Croizier *et al*., 1994) or from transfected plasmids (Luckow, 1991; Xiong *et al*., 1991). Other changes include reiterations of viral DNA (Burand & Summers, 1982; Carstens & Lu, 1990; Lee &Krell, 1994). These genetic exchanges or rearrangements could dramatically change the virus by altering its virulence or even host range as demonstrated for an AcMNPV which exchanged as little as 79 bp of the *Bombyx mori* NPV (BmNPV) helicase gene, allowing AcMNPV to replicate in *B. mori* cells (Croizier *et al*., 1994).

Passage, genotypic changes and the few polyhedra (FP) phenotype

The serial passage of any virus results in the accumulation of genotypic variations among any virus population, including that of baculoviruses. One of the first variations which arose during serial passage of baculoviruses described at the genetic level, was a change from the parental, many polyhedra per cell (MP) phenotype, to the few polyhedra per cell (FP) phenotype reported for both AcMNPV and *Galleria mellonella* NPV (GmNPV; Fraser, 1987; Fraser *et al*., 1983, 1985; Wang *et al*., 1989) and TnNPV (Potter *et al*., 1976). That this was the earliest and most common phenotypic change during serial passage (Kumar & Miller, 1987) reflects the high frequency of genetic alterations in the FP-locus and the non lethal nature of this change for virus grown in tissue culture.

Potter *et al*. (1976) showed for TnNPV that FP plaques could be recovered as early as passage 9 of an undiluted serial passage in TN–368 cells. The proportion of FP plaques recovered increased during passage so that by passage 14, no MP plaques were observed. Also non occluded virus (NOV; i.e., extracellular virus) from the FP isolates were released at a faster rate than MP PIB. Although FP NOV were infectious to TN–368 cells and to insects when injected into the haemocoel, the corresponding FP polyhedra were much less infectious *per os* than the MP NOV. When TnNPV MP NOV was passaged by injection into *T. ni* larvae, FP NOV was observed by 4 passages and by passage 14 represented about 95% of plaques isolated from infected haemolymph (Potter *et al*., 1978). FP virus was detected only sporadically for virus passaged as PIB and *per os* infection of larvae, but was not maintained nor was there a change in virulence in *per os* transmitted virus. Thus one way to maintain the MP character of an inoculum is to ensure that it is passaged as PIB through insect larvae.

The generation of some AcMNPV FP mutants occurs even during a single passage at low moi and most are due to transposon insertion in the FP-locus (Miller & Miller, 1982; Fraser, 1987). The very high frequency of FP mutants from several insect cells and insect species suggested that transposon mutagenesis was common. From the first description of the FP plaques by Hink & Vail (1973), the incidence of FP and their molecular basis have been of major interest. Miller & Miller (1982) in their analysis of a 7.3 kb insertion in the genome of an FP mutant (FP-DL) later established the role of transposon-mediated mutagenesis in the generation of some FP mutations. For AcMNPV, FP mutants were detected within only one or two passages in TN–368 cells (Hink & Strauss, 1976). FP virus was also detected *in vivo* from *per os* (PIB) infected *T. ni*, *Heliothis virescens*, *Helisidota caryae*, and *S. frugiperda* larvae. For these, the LD_{50} of the parental MP PIB was 38 times higher than for FP PIB. MP virus could be "rescued" (from 21 to 67%) if a predominantly FP virus preparation was passed *in vivo* (*per os*) through *T. ni* larvae. Host DNA insertions of 0.8 to 2.8 kb were detected in the genomes of AcMNPV and GmNPV FP mutants (Fraser, 1987; Fraser *et al*., 1983)

Fraser & Hink (1982) first demonstrated how fast the passage effect can manifest itself in the generation

of FP virus. The FP phenotype was detected within two passages of GmNPV in TN–368 cells or in *G. mellonella* larvae. The proportion of FP virus was virtually 100% by the 10th passage in TN–368 cells or by 5 serial injection passages in *G. mellonella* larvae. GmNPV FP virus is a stable genotypic variant which was maintained through at least 10 serial injection passages in *G. mellonella* larvae. The MP virus always generated both MP and FP virus whether passaged through TN–368 cells or by injection through *G. mellonella* larvae. Thus the FP virus resulted from frequent spontaneous mutations of a single MP virus rather than the FP virus being present in the original MP virus preparation. For GmNPV, the FP virus was up to 350 fold less infectious than the parental MP virus.

Genomic changes and serial passage

Wickham *et al.* (1991) followed the effect of serial passage and moi on genomic changes in a recombinant, β-gal AcMNPV in Sf21 cells. Virus production decreased from 814 pfu/cell for the original isolate to 35 pfu/cell for a late passage isolate. DIP at late passage were detected by electron microscopy showing up to 60% short (200 nm) particles. The DIP genome had a deletion of contiguous *Eco*R1 A, I, J, K, N, and O fragments which includes the β-gal gene and corresponded to the region that Kool *et al.* (1991) reported was missing in their DIP (Figure 1E). The DIP preparation also interfered with replication of the original β-gal AcMNPV. When DIP-containing virus (moi of 100) was added, the amount of β-gal AcMNPV produced per cell decreased from 814 pfu/cell (without DIP) to 40 pfu/cell. However the percent of standard virus in the DIP preparation increased from 15 to 65% after a single passage at a much reduced moi of 0.001. While the production of β-gal per cell decreases due to the DIP, this effect is less obvious at low moi. It is unfortunate that Wickham *et al.* (1991) did not give sufficient details (e.g., initial moi, frequency of passaging, passage number analyzed) so that their results could be compared to those of Kool *et al.* (1991) and others.

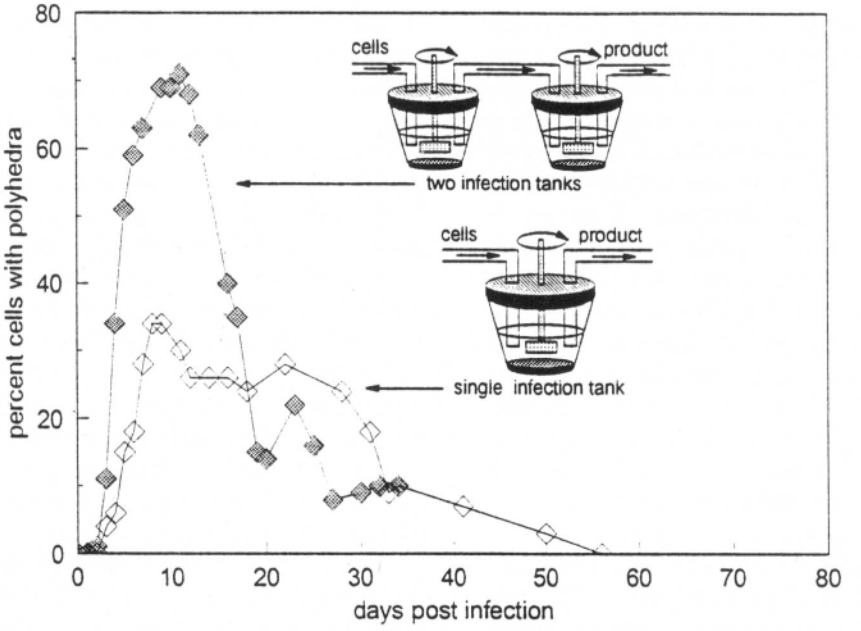
In a more systematic study, Kool *et al.* (1993) followed HMP of AcMNPV in Sf21 cells through 40 passages in culture flasks. As for studies of virus in bioreactors (Kompier *et al.*, 1988) most cells infected with P40 virus no longer produced polyhedra and the yield of NOV was about 100 fold lower than that of the inoculum. Much of the genome was deleted in late passage DIP and seven novel and hypermolar AcMNPV-specific *Eco*R1 fragments were detected within cells infected with P40 virus and in P41 NOV preparations. Since these fragments supported infection-dependent DNA replication, Kool *et al.* (1993) suggested that they contain *cis* sequences important for DNA replication and would have a selective advantage over the replication of the larger standard virus genome.

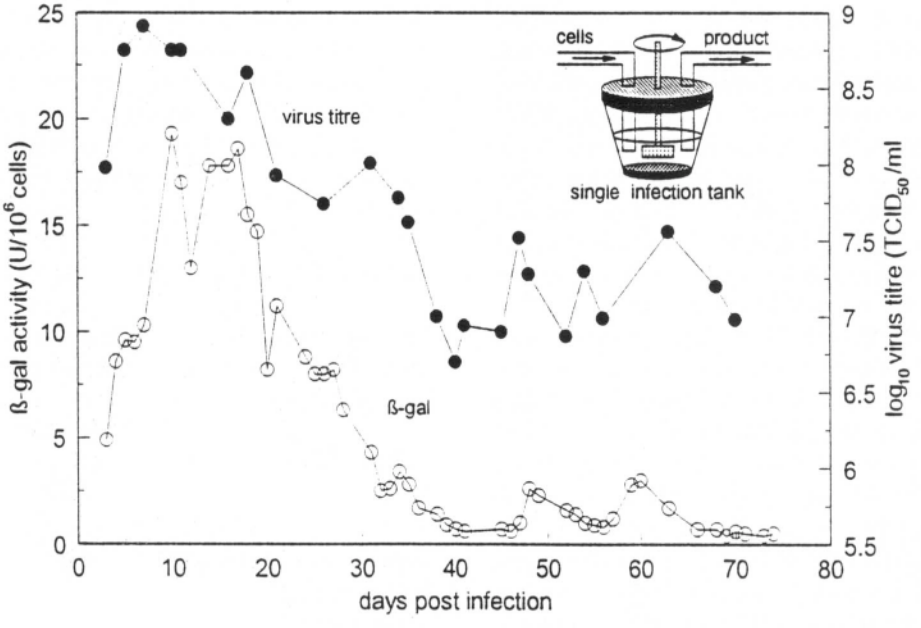
In a more extended study, Lee & Krell (1992, 1994) followed the generation of defective genomes during HMP up to passage 81 (Figure 1). Like Kool *et al.* (1993) the region from 1.7 to 45.0 m.u. was deleted within 40 passages in the defective genomes. The size of the defective genomes became smaller during passage down to an average size of about 50 kb. Up to passage 81 even more of the genomic DNA genome was deleted although the overall size of the genome was not reduced any further. By passage 80 only about 2.8 kb of the standard genome (m.u. 85.0 to 87.2; Figure 1) was maintained in the defective genomes isolated from P80 infected cells and from P81 NOV. This short sequence was reiterated and originated from the AcMNPV *Hin*dIII K region which Lee & Krell (1994) suggest contains an origin of DNA replication. Because of the smaller size and presence of multiple origins such genomes could compete for essential *trans*-acting factors such as DNA polymerase and helicase and would replicate at the expense of the standard genome.

Passage effect and bioreactors

For large scale production of foreign proteins at economical levels cells have to be grown and infected in bioreactors where cell growth, virus infection and foreign protein synthesis occur simultaneously within the same chamber. Under a continuous system the virus undergoes many passages over a short period of time. Virus grown in flasks undergoes only a single replication cycle and virus is normally passaged about every 3 or more days. However, in a bioreactor where uninfected cells are being continuously supplied, virus is "passaged" after each replication cycle time (e.g., 8–24 hr). In the bioreactor setting, since the virus is being passaged at a high frequency and at high moi the consequences of the passage effect are noted within about two weeks (Figure 2).

In one of the first systematic evaluations of the passage effect on wild type AcMNPV in a bioreactor, Kompier *et al.* (1988) followed the production of a standard virus (AcMNPV-E2 in Sf21 cells) in a


percent cells with polyhedra
days post infection
cells
product
two infection tanks
single infection tank


ß-gal activity (U/10^6 cells)
log10 virus titre (TCID50/ml
days post infection
virus titre
ß-gal
cells
product
single infection tank

two stage bioreactor. The first continuous stirred tank reactor (CSTR I, 0.8 1 of medium) was for growth of cells which were fed continuously to the second virus infection tank (CSTR II, 0.8 1 of medium, with a residency time of 60 hr). Over the first 25 days of continuous production they obtained close to the predicted percent of virus infected cells (65% vs. 68%) with a yield of 10^7 polyhedra and 10^8 infectious NOV per ml (equivalent to 25 polyhedra and 250 NOV per infected cell). Thereafter however, productivity declined so that by day 32 pi, only 25% of cells were infected (contained polyhedra). By day 56 pi, less than 5% of cells showed any evidence of polyhedra, the total number of polyhedra was near zero or only aberrant polyhedra were detected, and the infectious NOV titre was reduced to 10^5 per ml. From this van Lier *et al.* (1990a; 1990b) altered the reactor design to have two half-volume infection reactors connected in series to achieve a better residence time of 30 hr and an initial infection level of up to 81% infected cells. Under pseudo steady state conditions during the first 15 days pi, the proportion of AcMNPV-infected cells in the first and second infection tanks were 30–35% and 50–70% respectively, close to the theoretical levels of 45 and 81% respectively, and was greater than the 26% level in the single infection tank system (Figure 2). While the two infection tank system allowed for greater virus production, the negative aspects of the passage effect occurred much earlier, by about 15 days pi. The passage effect was more noticeable in the second of the two infection tanks such that by 20 days pi, there was a reduction in the polyhedral yield (from 1.2×10^7 to 1×10^6 **PIB** ml^{-1}), in the number of polyhedra per cell (from 55 to 7 per cell) and in the percent of infected cells (from 70 to 10%; Figure 2). Moreover, the titre of infectious NOV decreased 100 fold over this 20 day period. This loss of overall productivity coincided with accumulation of later passage virus. By mathematical modelling van Lier *et al.* (1992) and Kool *et al.* (1990) showed that in the two infection tank system, at any given time pi, the passage number frequency profile was skewed to a greater proportion of later passage virus compared to the single infection tank system. For example, in the two infection tank system the 11th passage virus population was dominant by 15 days pi whereas for the single infection tank system it was not dominant until about 25 days pi. Unfortunately they did not compare the cumulative yield and relative production costs between the two stage and three stage bioreactor systems. Nevertheless their results from a bioreactor mimic the changes reported by Wickham *et al.* (1991) for serially passaged recombinant virus.

Since bioreactors are also needed for production of foreign proteins produced by recombinant baculoviruses it was important to study the kinetics of such production in bioreactors. Kool *et al.* (1991) & van Lier *et al.* (1992) followed the production of β-galactosidase by a recombinant AcMNPV producing β-gal (**Acβgal**) in a bioreactor with a single infection reactor tank (Figure 2). Production increased to a maximum of about 15 β-gal **U/10^6** cells but by 20 days pi only half this amount was produced and by 40 days pi it was only about 0.5 **U/10^6** cells and the virus titre decreased from 10^9 **$TCID_{50}$** ml^{-1} to 10^7 **$TCID_{50}$** ml^{-1}. At 48 and 60 days pi there was a slight increase in the level of β-gal (3 **U/10^6** cells). This coincided with peaks in the NOV titre reflecting the "von Magnus" phenomenon and attributed to the generation and passaging of DIP (Huang & Baltimore, 1977).

Kool *et al.* (1991) were the first to demonstrate the production of DIP during passage of a recombinant virus, **Acβgal,** in a bioreactor. The decreased level of **β-gal** production in a bioreactor after about 2 weeks of culture was coincident with the detection of shorter than normal virions (190 nm, compared to 330 nm for standard NOV; Figure 1). Loss of β-gal production also correlated with the presence of submolar AcMNPV DNA fragments representing a viral DNA population with 43% of the genome (1.7 to 45.0 m.u.) being deleted. Much of the sequence that was deleted was contiguous and included the β-gal gene region. The shorter virus needed helper standard virus to replicate and could also interfere with standard virus replication and expression. Because of the smaller nucleocapsid size, reduced genome size and interfering characteristics of these shorter NOV Kool *et al.* (1991) concluded that they were DIP. Because the proportion of DIP increases with late passage and concomitant high moi in a bioreactor, their influence in the passage effect for both wild type virus and recombinant virus producing a foreign protein is detected even early (within weeks) in a bioreactor cycle. In a bioreactor, the decreased yield of infectious virus and lower yield of β-gal would be due to both the interference by the DIP and loss of the β-gal coding region in the DIP genome.

In order to better evaluate and model the passage effects observed for polyhedra production and production of recombinant virus encoded foreign proteins, De Gooijer *et al.* (1992) developed a structured dynamic model for use in bioreactor systems. This model recognized the importance, not only of DIP, but also

of non-infectious virus resulting in abortive infection. They developed a series of equations to calculate the fraction of noninfected cells (F_o), abortively infected cells (F_a), successfully infected cells (F_i) and defectively (i.e. infected with DIP) infected cells (F_d). The equations were based on certain assumptions such as the number of effective virus receptors and variations in the number and ratios of the three types of virions. Among additional assumptions were that DIP were produced two hours earlier than infectious NOV in the same cells and that because of their smaller genome had a selective replicative advantage. From their equations they determined a set of parameters (some already predefined such as starting cell and virus titres and moi) which closely mimicked the results obtained under published experimental conditions. The passage effect and the frequency distribution of virus passage numbers with time pi was found to be an important predictor of bioreactor productivity. From their model they also suggested, as a partial solution to the passage effect, setting up a "repeated batch infection" in which, at certain intervals (e.g., every 5 days), most (97%) of the infection tank contents would be removed and replaced with new cells. This would remove most of the accumulated DIP and dilute the remaining DIP to a low enough level that they have less of an influence. Use of this and other mathematical approaches such as the Taguchi method have already been applied to improve the baculovirus expression system (Burch *et al*., 1995; Zhang *et al*., 1995). These can provide testable models and bioreactor design and operation can be modified (e.g., Kioukia *et al*., 1995) to reduce passage effects and improve virus or foreign protein production.

Viral persistence

In some cases virus passage and concomitant generation of DIP could result in persistently infected cells. The cell line IMC-Hz–1 from *H. zea* is persistently infected with the *H. zea* baculovirus, Hz–1, and on occasion, undergoes a crisis and shows a cytopathic effect (Granados *et al*., 1978; Wood & Burand, 1986). Huang *et al*. (1982) were the first to show that the genome of uncloned Hz–1 consisted of standard virus size DNA (228 kb) and smaller DNAs down to about 100 kb. Later, Burand *et al*. (1983), Burand & Wood (1986) and Chao *et al*. (1990) mapped the Hz–1 and Hz–1 DIP genomes and showed that the DIP genomes represented deletions ranging from 17 to up to 130 kb. The virus from these cells were infectious to at least four other insect cell lines; TN– 368, Sf21, 1075 (from *H. zea*) and IPLB–652 (*Porthetria dispar*). Virus from the original IMC-Hz–1 cell line could also produce persistently infected TN–368 cells but not two clonal isolates of TN– 368 (Ralston *et al*., 1981). Burand *et al*. (1983) showed that Hz–1 from persistently infected cells contained shorter nucleocapsids (376 ± 76 nm) than those of standard virus (414 ± 30 nm) suggesting the presence of DIP. The persistently infected TN–368 cells, like IMC-Hz–1, also went through a crisis phase, usually before passage 25. Surviving cells shed virus at about 10^4 pfu ml^{-1} at least up to passage 100. McIntosh & Ignoffo (1981) demonstrated that a persistently infected Sf21 cell line (SFP) could be established by culturing cells which remained attached following infection with SfNPV at an moi of 4 $TCID_{50}$/cell. These SFP cells continued to produce virus but at a 100 fold lower level, were refractory to infection with the homologous virus and interfered with replication of AcMNPV. Both the virus and polyhedra that were produced in persistently infected cells were less infectious than standard SfNPV. Viral transcription in persistently infected TN– 368 (TNP3) and Sf21 (SFP2) cells was restricted to a single 2.9 kb transcript (PAT1; Chao *et al*., 1992) and virus specific protein synthesis in cells infected with Hz–1 DIP was delayed and was at reduced levels compared with standard virus (Burand & Wood, 1986). Persistently infected IMC-Hz–1 cells were resistant to superinfection by Hz–1 but were susceptible to infection by AcMNPV. These studies suggested that although persistent infections could be established for some baculoviruses this might be somewhat host specific.

Since at least two viruses can generate persistently infected cells, this is one possible consequence of passage of the virus. Such cells could outgrow uninfected cells (McIntosh & Ignoffo; 1981) and therefore could reduce the virus yield (and expression of foreign protein for recombinant baculoviruses). Since these cells also produce DIP this would further compromise the ability of such cultures to produce infectious virus. Although it has not yet been reported that persistently infected cells arise in bioreactors, this is one possibility that should be evaluated.

Virus passage in insects and viral fitness

Three phenotypic variants, Ld-S, Ld-F and Ld-V with several, few and variable numbers of polyhedra per cell respectively were identified in *Lymantria dispar*

NPV preparations (Cusack & McCarthy, 1989). Of these, HMP of Ld-S in Ld–652Y cells led to a reduced number of polyhedra per cell and by passage 20 many cells exhibited viral cytopathic effect but no polyhedra. The DNA from P20 virus contained additional viral DNA, and submolar fragments suggesting that HMP of Ld-S resulted in the generation of DIP which decreased the production of infectious polyhedra. LdMNPV was also passaged by isolating virus (LdMNPV-Ab-a624) from occlusion bodies and infecting LdFBc1 cells at each passage (Lynn, 1994). Although the infectivity of the alkali-liberated occluded virus (ALOV) was lower than for NOV, the amount of PIB produced over 15 serial passages with ALOV was 50 fold higher than that for NOV. Less than 5% FP plaques were observed for ALOV-passaged virus. Thus ALOV could be used as inoculum to reduce the incidence and selection of FP virus. This may be practical for initial infections or for passage in flasks but might be impractical in a bioreactor.

Fuxa & Richter (1992) followed the vertical transmission and effectiveness of SfNPV after initial passage through *S. frugiperda* pupae. Vertical transmission of standard SfNPV showed only 13.8 to 3.9% mortality from the first to the fifth generation of the initially infected insect. The vertically transmitted SfNPV selected from pupae, however, showed a mortality of 18.8% in the first generation to 12.2% by the fifth generation. In this example, the passage effect for virus passaged in pupae selected for viruses with enhanced, but limited, vertical transmission properties.

Passage of virus through alternate hosts or by different routes can also result in changes in baculoviruses. BmNPV PIB from the original host *B. mori*, when passaged through the rice stem borer, *Chilo suppressalis* (Watanabe *et al*., 1975) became cuboidal and otherwise changed morphology with increasing passage numbers (up to 25) and fewer singly enveloped virions became occluded. Even the route of passage is critical in the selection of dominant baculovirus genotypes (Fraser & Hink, 1982). When a mixture of MP and FP virus was passaged through *G. mellonella* larvae by feeding PIBs, the MP genotype dominates. If the same mixture is passaged by injection of NOV into the insect haemocoel (or inoculation of tissue culture cells) the FP genotype dominates. Clearly in this example (and others; Hink & Strauss, 1976), the MP genotype, which produces more infectious PIBs than the FP genotype (Ramoska & Hink, 1974; Fraser & Hink, 1982; Potter *et al*., 1976), has an enhanced replication potential when fed as PIB. In contrast, when the PIB intermediate is avoided by direct injection of NOV, the FP genotype is favoured (presumably because it produces more NOV than the MP virus; Potter *et al*., 1976; Fraser & Hink, 1982). Similarly five passages of an isolate of *H. zea* NPV through *H. zea* larvae by PIB increased the infectivity of the resultant PIB by about 80% and was stable for the next 11 passages (Shapiro & Ignoffo, 1970).

One factor which might influence the outcome of the passage effect in cultures infected with a mixed genotype virus preparation is their relative viral fitness. Many genotypes emerge and compete during virus passage (e.g., DIP, MP and FP plaque variants). For genetically engineered virus used in biocontrol, the virus strain selected may be co-occluded with the standard virus (Wood & Granados, 1991) to allow for co-infection of insects with both viruses. Even if a genetically engineered virus is applied alone, there is the possibility of co-infection with indigenous viruses. Such multiple infections result in a competition for the most fit virus after several passages in either cell cultures (e.g., bioreactors) or *in vivo* (natural populations of susceptible larvae). Huang *et al*. (1991) followed the serial passage of a mixed infection of wild type AcMNPV with an AcMNPV recombinant (Ac360-β-gal) and showed that the wild type strain attained a 1.7 fold higher titre and was clearly dominant. The initial relative proportion of the two viruses did not influence the outcome after about 20 passages, nor was there any change in total virus production. Their data suggest that recombination between viruses was of little importance and that the wild type was still more fit in the mixed infection.

Concluding remarks

It should be clear from this review that the passage effect for serially passaged baculovirus in insect tissue culture cells and in insect haemolymph and especially in bioreactors, adversely affects virus integrity, infectivity and, for recombinant baculoviruses, production of foreign proteins. During passage, many minor to major alterations occur in the viral genome due to its inherent genetic plasticity and these accumulate with each passage. In addition to inactivation or loss of genes for structural, regulatory and other essential functions in still viable virus, passage also leads to generation of DIP which replicate at the expense of standard virus. Although not yet reported for bioreactors there is also the potential to generate persistently infect-

ed cells which would decrease virus yield and expression, although this may be host cell and virus species dependent. Given the propensity of baculoviruses to undergo extensive genomic alterations and produce DIP during passage, it might seem a daunting task to suggest approaches to ameliorate these negative consequences. For batch production it is fairly easy to use low moi and early passage virus to reduce DIP production and accumulation of mutant viruses. However in bioreactors the major problem with passage is the concomitant high moi and consequent development of DIP. Bioreactors could be redesigned to maintain a low moi throughout the production cycle by approaches like that suggested by De Gooijer *et al.* (1992). Alternative models could develop systems for intermittent (or continuous) removal or inactivation of most of the virus that is produced in a bioreactor by using, for example, virus specific-antibody or flocculating agents. With some of the elegant modelling systems for bioreactor productivity, the passage number virus which is predominant at the time productivity starts to decline could be determined. From that it should be possible to design reactor conditions (double/triple infection tanks) or develop other strategies to optimize production up to the time when that passage number would become predominant. Since it seems inevitable that DIP will appear, one consideration would be to clone the foreign gene into DNA sequences which are maintained by and would be expressed by the DIP although there is no guarantee that such foreign gene sequences would not also be deleted from the DIP genome during passage.

In some cases baculoviruses would be produced for use as insecticides. For these there is the additional constraint that the virus produced would have to be virulent and in the form of PIBs. This is more difficult to achieve in a bioreactor or even in batch culture since some of the genes (e.g., polyhedrin, *egt*, MP) which appear to be inactivated even at early passage are required for virus occlusion and virulence in insects. Nevertheless, as shown in some of the studies involving *in vivo* passage through insects, virus inoculum passed initially in insects is preferable to tissue culture-derived virus for amplification in tissue culture. It would also be prudent to determine the time (and corresponding passage number distribution) during production in a bioreactor that virulence drops below an economic threshold and to redesign the bioreactor accordingly. It may still be necessary, although not always feasible, to passage and grow virus in insects at least for part of the production cycle, to maintain virulence.

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References

Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M & Possee RD (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202: 586–605.

Bangham CM & Kirkwood TBL (1990) Defective interfering particles: Effects in modulating virus growth and persistence. Virology 179: 821–826.

Beames B & Summers MD (1988) Comparisons of host cell DNA insertions and altered transcription at the site of insertions in few polyhedra baculovirus mutants. Virology 162: 206–220.

Beames B & Summers MD (1990) Sequence comparison of cellular and viral copies of host cell DNA insertions found in *Autographa californica* nuclear polyhedrosis virus. Virology 174: 354–363.

Blissard GW & Rohrmann GF (1990) Baculovirus diversity and molecular biology (Ann. Rev. Entomol. 35: 127–155.

Brown SE, Maruniak JE & Knudson DL (1985) Baculovirus (MNPV) genomic variants: characterization of *Spodnptera exempta* MNPV DNAs and comparison with other *Autographa californica* MNPV DNAs. J. Gen. Virol. 66: 2431–2441.

Burand JP & Summers MD (1982) Alteration of *Autographa californica* nuclear polyhedrosis virus DNA upon serial passage in cell culture. Virology 119: 223–229.

Burand JP & Wood HA (1986) Intracellular protein synthesis during standard and defective Hz–1 virus replication. J. Gen. Virol. 67: 167–173.

Burand JP, Wood HA & Summers MD (1983) Defective particles from a persistent baculovirus infection in *Trichoplusia ni* tissue culture cells. J. Gen. Virol. 64: 391–398.

Burch GJ, Ferguson CHE, Cartwright G & Kwong FYP (1995) Application of Taguchi experimental design to the optimisation of a baculovirus expression system. Biochem. Soc. Trans. 23: 107S.

Carstens EB (1982) Mapping the mutation site of an *Autographa californica* nuclear polyhedrosis virus polyhedron morphology mutant. J. Virol. 43: 809–818.

Carstens EB (1987) Identification and nucleotide sequence of the regions of *Autographa californica* nuclear polyhedrosis virus genome carrying insertion elements derived from *Spodoptera frugiperda.* Virology 161: 8–17.

Carstens EB & Lu A (1990) Nucleotide sequence and transcriptional analysis of the *Hin*dIII P region of a temperature-sensitive mutant of *Autographa californica* nuclear polyhedrosis virus. J. Gen. Virol. 71: 3035–3040.

Carstens EB, Lin-Bai Y & Faulkner P (1987) A point mutation in the polyhedrin gene of a baculovirus, *Autographa californica* MNPV, prevents crystallization of occlusion bodies. J. Gen. Virol. 68: 901–905.

Carstens EB, Williams GV, Faulkner P & Partington S (1992) Analysis of polyhedra morphology mutants *of Autographa californica* nuclear polyhedrosis virus: molecular and ultrastructural features. J. Gen. Virol. 73: 1471–1479.

Cary LC, Goebel M, Corsaro BG, Wang H-G, Rosen E & Fraser MJ (1989) Transposon mutagenesis of baculoviruses: Analysis *of Trichoplusia ni* transposon IFP2 insertions within the FP- locus of nuclear polyhedrosis viruses. Virology 172: 156–169.

Chao Y-C, Hamblin M & Wood HA (1990) Physical map of Hz–1 baculovirus genome from standard and defective interfering particles. J. Gen. Virol. 71: 1265–1270.

Chao Y-C, Wood HA, Chang C-Y, Lee H-J, Shen W-C & Lee H-T (1992) Differential expression of Hz–1 baculovirus genes during productive and persistent infections. J. Virol. 66: 1442–1448.

Croizier G, Croizier L, Argaud O & Poudevigne D (1994) Extension *of Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. Proc. Natl. Acad. Sci. USA 91: 48–52.

Croizier G, Croizier L, Baiche G & Chaufaux J (1985) Évolution de la composition génétique et du pouvoir infectieux du baculovirus de *Mamestra brassicae* L. au cours de 25 multiplications successives sur les larves de la noctuelle du chou. Entomophaga 30: 365–374.

Cusack T & McCarthy WJ (1989) Effect of serial passage on genetic homogeneity of a plaque variant of *Lymantria dispar* nuclear polyhedrosis virus (Hamden LDP–67). J. Gen. Virol. 70: 2963–2972.

De Gooijer CD, Koken RHM, van Lier FLJ, Kool M, Vlak JM & Tramper J (1992) A structured dynamic model for the baculovirus infection process in insect-cell reactor configurations. Biotechnol. Bioengin. 40: 537–548.

Duncan R, Chung KL & Faulkner P (1983) Analysis *of Autographa californica* nuclear polyhedrosis virus with a defect in the morphogenesis of the occlusion body macromolecular lattice. J. Gen. Virol. 64: 1531–1542.

Faulkner P (1981) Baculovirus. In: Pathogenesis of Invertebrate Microbial Disease. Davidson E (ed.) (pp. 3–37) Osmun & Co., New York.

Fraser MJ (1987) FP mutation of nuclear polyhedrosis viruses: a novel system for the study of transposon-mediated mutagenesis. In: Maramorosch K (ed) Biotechnology in Invertebrate Pathology and Cell Culture, (pp. 265–293) Academic Press, New York. Fraser MJ & Hink WF (1982) The isolation and characterization of the MP and FP plaque variants of *Galleria mellonella* nuclear polyhedrosis virus. Virology 117: 366–378.

Fraser MJ, Brusca JS, Smith GE & Summers MD (1985) Transposon-mediated mutagenesis of a baculovirus. Virology 145: 356–361.

Fraser MJ, Smith GE & Summers MD (1983) Acquisition of host cell DNA sequences by baculoviruses: relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. J. Virol. 47: 287–300.

Friesen PD & Nissen MS (1990) Gene organization and transcription of TED, a lepidopteran retrotransposon integrated within the baculovirus genome. Mol. Cell. Biol. 10: 3067–3077.

Friesen PD, Rice WC, Miller DW & Miller LK (1986) Bidirectional transcription from a solo long terminal repeat of the retrotransposon TED: Symmetrical RNA start sites. Mol. Cell Biol. 6: 1599–1607.

Fuxa JR & Richter AR (1992) Virulence and multigeneration passage of a nuclear polyhedrosis virus selected for an increased rate of vertical transmission. Biological Control 2: 171–175.

Gombart AF, Blissard GW & Rohrmann GF (1989) Characterization of the genetic organization of the *Hin*dIII M region of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* reveals major differences among baculoviruses. J. Gen. Virol. 70: 1815–1828.

Granados RR, Nguyen T & Cato B (1978) An insect cell line persistently infected with a baculovirus-like particle. Intervirol. 10: 309–317.

Hink WF & Strauss E (1976) Replication and passage of alfalfa looper nuclear polyhedrosis virus plaque variants in cloned cell cultures and larval stages of four host species. J. Invertebr. Pathol. 24: 49–55.

Hink WF & Vail PV (1973) A plaque assay for titration of alfalfa looper nuclear polyhedrosis virus in a cabbage looper (TN–368) cell line. J. Invertebr. Pathol. 22: 168–174.

Huang AS & Baltimore D (1977) Defective interfering animal viruses. In: Fraenkel-Conrat H & Wagner RR (eds.) Comprehensive Virology Vol. 10 Regulation and Genetics. (pp. 73–116) Plenum Press, New York.

Huang Y-S, Bobseine KL, Setzer RW & Kawanishi CY (1991) Selection kinetics during serial cell culture passage of mixtures of wild-type *Autographa californica* nuclear polyhedrosis virus and its recombinant **Ac360-β-gal**. J. Gen. Virol. 72: 2653–2660.

Huang Y-S, Hedberg M & Kawanishi CY (1982) Characterization of the DNA of a nonoccluded baculovirus Hz–lV. J. Virol. 43: 174–181.

Kioukia N, Nienow AW, Emery AN & Al-Rubeai M (1995) Physiological and environmental factors affecting the growth of insect cells and infection with baculovirus. J. Biotechnol. 38: 243–251.

Knell JD & Summers MD (1981) Investigation of genetic heterogeneity in wild isolates of *Spodoptera frugiperda* nuclear polyhedrosis virus by restriction endonuclease analysis of plaque-purified variants. Virology 112: 190–197.

Kompier R, Tramper J & Vlak JM (1988) A continuous process for the production of baculovirus using insect-cell cultures. Biotechnol. Lett. 10: 849–854.

Kool M, van den Berg PMMM, Tramper J, Goldbach RW & Vlak JM (1993) Location of two putative origins of DNA replication of *Autographa californica* nuclear polyhedrosis virus. Virology 192: 94–101.

Kool M, van Lier FLJ, Vlak JM & Tramper J (1990) Production of (recombinant) baculoviruses in a two-stage bioreactor system using insect-cell cultures. In: Dekkers JJ, van der Plas HC & Vuijk DH (eds.) Agricultural Biotechnology in Focus in the Netherlands. (pp. 64–70) Pudoc, Wageningen.

Kool M & Vlak JM (1993) The structural and functional organization of the *autographa californica* nuclear polyhedrosis virus genome. Arch. Virol. 130: 1–16.

Kool M, Voncken JW, van Lier FLJ, Tramper J & Vlak JM (1991) Detection and analysis *of Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology 183: 739–746.

Kumar S & Miller LK (1987) Effects of serial passage of *Autographa californica* nuclear polyhedrosis virus in cell culture. Virus Res. 7: 335–349.

Lee HY & Krell PJ (1992) Generation and analysis of defective genomes of *Autographa californica* nuclear polyhedrosis virus. J. Virol. 66: 4339–4347.

Lee HY & Krell PJ (1994) Reiterated DNA fragments in defective genomes of *Autographa californica* nuclear polyhedrosis virus are competent for AcMNPV-dependent DNA replication. Virology 202: 418–429.

Lee HH & Miller LK (1978) Isolation of genotypic variants of *Autographa californica* nuclear polyhedrosis virus. J. Virol. 27: 754–767.

Lu A & Carstens EB (1991) Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis virus. Virology 181: 336–347

Luckow VA (1991) Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: Pokop A, Bajpai RK & C Ho C (eds.) Recombinant DNA Technology and Applications. (pp. 97–152) McGraw-Hill, New York.

Lynn DE (1994) Enhanced infectivity of occluded virions of the gypsy moth nuclear polyhedrosis virus for cell cultures. J. Invertebr. Pathol. 63: 268–274.

MacKinnon EA, Henderson JF, Stoltz DB & Faulkner P (1974) Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro*. J. Ultrastruct. Res. 49: 419–435.

Maeda S, Mukohara Y & Kondo A (1990) Characteristically distinct isolates of the nuclear polyhedrosis virus from *Spodoptera litura*. J. Gen. Virol. 71: 2631–2639.

McIntosh AH & Ignoffo CM (1981) Establishment of a persistent baculovirus infection in a lepidopteran cell line. J. Invertebr. Pathol. 38: 395–403.

McIntosh AH, Rice WC & Ignoffo CM (1987) Genotypic variants in wild-type populations of baculoviruses. In: Maramorosch K (ed.) Biotechnology in Invertebrate Pathology and Cell Culture. (pp. 305–325) Academic Press, New York.

Miller LK (1984) Exploring the gene organization of baculoviruses. Methods in Virology 7: 227–258.

Miller DW & Miller LK (1982) A virus mutant with an insertion of a copia-like transposable element. Nature 299: 562–564.

O'Reilly DR & Miller LK (1990) Regulation of expression of a baculovirus ecdysteroid UDPglucosyltransferase gene. J. Virol. 64: 1321–1328.

O'Reilly DR, Passarelli AL, Goldman IF & Miller LK (1990) Characterization of the DA26 gene in a hypervariable region of the *Autographa californica* nuclear polyhedrosis virus genome. J. Gen. Virol. 71: 1029–1037.

Payne CC (1988) Pathogens for the control of insects: where next?. Phil. Trans. R. Soc. Lond. B 318: 225–248

Potter KN, Faulkner P & MacKinnon EA (1976) Strain selection during serial passage of *Trichoplusia ni* nuclear polyhedrosis virus. J. Virol. 18: 1040–1050.

Potter KN, Jaques RP & Faulkner P (1978) Modification of *Trichoplusia ni* nuclear polyhedrosis virus passaged *in vivo*. Intervirol. 9: 76–85.

Ralston AL, Huang Y-S & Kawanishi CY (1981) Cell culture studies with the IMC-Hz–1 nonoccluded virus. Virology 115: 33–44.

Ramoska WA & Hink WF (1974) Electron microscope examination of two plaque variants from a nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica*. J. Invertebr. Pathol. 23: 197–201.

Roux L Simon AE & Holland JJ (1991) Effects of defective interfering viruses on virus replication and pathogenesis *in vitro* and *in vivo*. Adv. Virus Res. 40: 181–211.

Shapiro M & Ignoffo CM (1970) Nucleopolyhedrosis of *Heliothis:* activity of isolates from *Heliothis zea*. J. Invertebr. Pathol. 16: 107–111.

Summers MD, Smith GE, Knell JD & Burand JP (1980) Physical maps of *Autographa californica* and *Rachiplusia ou* nuclear polyhedrosis virus recombinants. J. Virol. 34: 693–703.

Tramper J & Vlak JM (1986) Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. Ann NY Acad Sci. 469: 279–288.

Van Lier FLJ, Kool M, van den End EJ, De Gooijer CD, Usmany M, Vlak JM & Tramper J (1990a) Production of baculovirus or recombinant derivatives in continuous insect-cell bioreactors. Ann NY Acad Sci 613: 183–190.

Van Lier FLJ, van den End EJ, De Gooijer CD, Vlak JM & Tramper J (1990b) Continuous production of baculovirus in a cascade of insect-cell reactors. Appl. Microbiol. Biotechnol. 33: 43–47.

Van Lier FLJ, van der Meijs WCJ, Grobben NG, Olie RA, Vlak JM & Tramper J (1992) Continuous β-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. 22: 291–298

Wang H-GH, Fraser MJ & Gary LC (1989) Transposon mutagenesis of baculoviruses: analysis of TFP3 lepidopteran transposon insertions at the FP locus of nuclear polyhedrosis viruses. Gene 81: 97–108.

Watanabe H, Aratake Y & Kayamura T (1975) Serial passage of a nuclear polyhedrosis virus of the silkworm *Bombyx mori*, in larvae of the rice stem borer, *Chilo suppressalis*. J. Invertebr. Pathol. 25: 11–17.

Weitzman MD, Possee RD & King LA (1992) Characterization of two variants of *Panolis flammea* multiple nucleocapsid nuclear polyhedrosis virus. J. Gen. Virol. 73: 1881–1886.

Wickham TJ, Davis T, Granados RR, Hammer DA, Shuler ML & Wood HA (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnol. Lett. 13: 483–488.

Wood HA & Burand JP (1986) Persistent and productive infections with the Hz–1 baculovirus. Curr Topics Microbiol and Immunol. 131: 119–133.

Wood HA & Granados RR (1991) Genetically engineered baculoviruses as agents for pest control, Ann. Rev. Microbiol. 45: 69–87

Xiong G, Schorr J, Tjia ST & Doerfler W (1991) Heterologous recombination between *Autographa californica* nuclear polyhedrosis virus DNA and foreign DNA in non-polyhedrin segments of the viral genome. Virus Res. 21: 65–85.

Zhang J, Kalogerakis N, Behie LA & latrou K (1994) Optimum injection conditions for recombinant protein production in insect cell (Bm5) suspension culture. Biotechnol. Prog. 10: 636–643.

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Post-translational modifications in insect cells

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Introduction

Maturation of many proteins, in particular of proteins entering the exocytotic transport route, involves post-translational processing. Most of these modifications are necessary for the biological functions of the proteins. It is therefore important to understand the capacity of insect cells to process proteins, when baculoviruses are used as expression vectors. The first membrane glycoprotein of vertebrate origin to be expressed in this system was the influenza virus hemagglutinin. I will focus here on the processing of the hemagglutinin in insect cells, but I will also refer to studies on other proteins.

Expression of the Hemagglutinin of Influenza Virus in Insect Cell Cultures and in Insect Larvae

The hemagglutinin of influenza A virus is a class I membrane glycoprotein whose structure has been analyzed in great detail (Wiley & Skehel, 1987). As the major viral antigen (Webster *et al.,* 1983), as the initiator of infection, and as an important determinant for virus pathogenicity (Klenk & Rott, 1988) it is of considerable biological interest. The biosynthesis of the hemagglutinin involves translation on membrane-bound polysomes and transport through the Golgi complex to the plasma membrane. At the co-translational level, the hemagglutinin is translocated into the lumen of the endoplasmic reticulum, freed of its amino-terminal signal peptide, and N-glycosylated with oligomannosidic side chains. Polypeptide folding and presumably formation of disulfide bonds begin already at this stage. The first posttranslational event is assembly of monomers to trimers that takes place either in the endoplasmic reticulum or in another pre-Golgi compartment. Trimers differ from monomers in protease sensitivity and in antigenic epitope pattern. Another early post-translational modification is attachment of fatty acids to carboxy-terminal cysteine residues. In the Golgi complex, oligosaccharides are trimmed and elongated to complex side chains. Finally, activation of the fusogenic activity of the hemagglutinin by proteolytic cleavage occurs either in the trans-Golgi compartment or in transport versicles on the way to the plasma membrane (for review see Nayak & Jabbar, 1989).

To express the hemagglutinin of fowl plague virus in insect cells a cDNA sequence of the hemagglutinin gene has been inserted into the BamHI site of the pAc373 polyhedrin vector. *Spodoptera frugiperda* (SF9) cells were cotransfered with this construct pAc-HA651 and authentic AcNPV DNA. Recombinant virus was selected by adsorption of transfected cells to erythrocytes followed by serial plaque passages on SF9 cells. When the insertion site of the hemagglutinin gene into the AcNPV genome was determined by restriction and Southern blot-hybridization analyses using hemagglutinin cDNA as probe, it was found to be located in the polyhedrin gene. Immunofluorescent labeling, immunoprecipitation, and Western blot analyses employing specific antisera revealed that SF9 cells produced hemagglutinin after infection with the recombinant virus (Kuroda *et al.*, 1986).

The hemagglutinin was also expressed in larvae of *Heliothis virescens* using the same vector. Animals were infected with the recombinant virus either by parenteral injection or by feeding. For the peroral uptake, recombinant virus occluded in polyhedra has been used

which was obtained from cultured SF9 cells after coinfection with authentic AcNPV. Immunohistological analyses of infected animals revealed that the hemagglutinin was expressed only in those tissues that are also permissive for the replication of authentic AcNPV. These tissues comprise hypodermis, fat body, and tracheal matrix. After peroral infection, hemagglutinin was also detected in individual gut cells. The amount of hemagglutinin synthesized in larvae after parenteral infection was 0.3% of the total protein, compared to 5% obtained in cultured insect cells. The hemagglutinin was transported to the cell surface and was expressed in polarized cells only at the apical plasma membrane (Kuroda *et al.*, 1989).

Oligomerization

There were differences, however, in the efficiency of hemagglutinin processing between SF9 and vertebrate cells. Centrifugation on sucrose density gradients and immune precipitation with conformation-specific antibodies were used to compare trimerization of the hemagglutinin in SF9 cells and in fowl plague virus infected MDCK cells. Trimerization of the hemagglutinin was incomplete in insect cells, and the kinetics of this reaction were about 3 times slower than in vertebrate cells. Similarly, post-translational proteolytic cleavage occurred in insect cells with a half time of 90 minutes, and a substantial fraction of the hemagglutinin persisted in uncleaved form. In contrast, hemagglutinin was almost completely cleaved in MDCK cells, and the half time of cleavage was only 30 min. The data indicate that in insect cells trimerization and, as a result, the subsequent processing steps of the hemagglutinin are retarded (Kuroda *et al.*, 1991).

Proteolytic Cleavage

Endoproteolytic cleavage, usually at arginine residues, is a common posttranslational modification of membrane and secretory proteins on the exocytotic transport route. Such proteins include precursors of peptide hormones, neuropeptides, growth factors, coagulation factors, serum albumin, cell-surface receptors, and adhesion molecules. All of these proteins play important roles in a large variety of different biological processes, and these functions depend on proteolytic cleavage of the proteins. The same type of processing has also been observed with many viral membrane proteins and, in some instances, proved to be a crucial factor in determining organ and host tropism, spread of infection, and pathogenicity of these viruses.

The enzymes responsible for the activation of these proteins often belong to a family of subtilisin-like eukaryotic endoproteases. Some of these proteases are constituents of the regulated secretory pathway and are, thus, present only in specific, e.g. neurosecretory, cells. Others are found in many cells as components of the constitutive secretory pathway. The prototype of the latter enzymes is furin which is ubiquitous in mammalian tissues. Furin is localized in the trans-Golgi network and has a substrate specificity for the amino acid sequence R-X-K/R-R at the cleavage site (for rev. see Klenk & Garten, 1994).

Fowl plague virus (FPV) is an influenza A virus with a hemagglutinin that is cleaved by furin (Stieneke-Gröber *et al.*, 1992). FPV hemagglutinin is activated in SF9 cells indicating that these cells contain a furin-like enzyme (Kuroda *et al.*, 1986). As already pointed out, however, cleavage occurs at a lower rate than in MDCK cells (Kuroda *et al.*, 1991). Inefficient cleavage in *Spodoptera frugiperda* cells has also been reported for other glycoproteins containing a furinspecific cleavage signal. Thus, the surface glycoprotein of rubella virus is cleaved quite slowly (Oker-Blom *et al.*, 1989). The fusion protein of human parainfluenza virus 3 does not show cleavage at all in these cells (Ray *et al.*, 1989). Inefficient cleavage has also been observed with the surface glycoprotein gp 160 of HIV (Hu *et al.*, 1987) and with the F protein of measles virus. The latter protein, however, was readily cleaved in a cell line derived from *Trichoplusia ni* (Vialard *et al.*, 1990). Different insect cells vary therefore in their proteolytic activity. Exact determination of the substrate specificity of these insect proteases has to wait until cDNA clones will be available which so far have been obtained only from *Drosophila melanogaster* tissues (Roebroek *et al.*, 1992, 1993).

Acylation

Proteins acylated in mammalian cells have also been found to contain palmitic acid in covalent hydroxylamine-sensitive linkage when expressed in *Spodoptera frugiperda* cells by baculovirus vectors. Thus, these cells have the capacity to acylate foreign proteins at cystein residues. Such proteins include HA-ras p21 (Page *et al.*, 1989), influenza virus hemagglutinin (Kuroda *et al.*, 1991), G proteins (Linder *et al.*,

1993), and the surface glycoprotein (GP) of Marburg-virus (Funke *et al*., 1995). In contrast to authentic Marburg virus GP, recombinant GP still retained a small fraction of labeled fatty acids after mercaptoethanol treatment at neutral pH. Complete removal was only obtained when this treatment was carried out at pH 10. This result suggests that GP contains also some fatty acid residues in oxyester linkages. Such linkages have also been assumed to be present in the 67k glycoprotein of AcMNPV (Roberts & Faulkner, 1989).

Membrane binding of proteins may also be mediated by glycosylphosphatidylinositol (GPI) anchors. One of these proteins is CD 59 antigen which protects human cells from complement-mediated lysis. Functionally active CD 59 was expressed from a baculovirus vector in *Spodoptera frugiperda* cells. The recombinant protein was released from the cell surface by treatment with phosphatidylinositol-specific phospholipase C, indicating that it was attached to the insect cell membrane via a GPI anchor (Davies & Morgan, 1993).

Glycosylation

Glycosylation is a common feature of most eucaryotic membrane and secretory proteins. The carbohydrate moiety contributes to important physical properties, such as conformational stability, protease resistance, charge, and hydrophilicity. Evidence has also been obtained indicating that the carbohydrate moieties of glycoproteins may function as recognition determinants in host-pathogen relationships, protein targeting, and cell-cell interactions (Elbein, 1987; Paulson, 1989; Rademacher *et al*., 1988). The biosynthesis of *N*-linked oligosaccharides involves assembly of an oligomannosidic precursor oligosaccharide linked to a dolicholpyrophosphate carrier and its transfer to the polypeptide. The further steps comprise trimming reactions yielding oligomannosidic carbohydrates with 5-8 mannose residues, and addition of *N*-acetylglucosamine, galactose, fucose, and neuraminic acid residues to complete the assembly of complex-type oligosaccharides. These reactions have been studied in great detail in mammalian and other vertebrate cells (Kornfeld & Kornfeld, 1985).

Although insect glycosylation has not been studied in such detail, studies on mosquito cells demonstrated that carbohydrates can be trimmed to truncated carbohydrates but are not elongated to complex side chains (Butters *et al*., 1981; Hsieh & Robbins, 1984). Studies on recombinant proteins from *Spodoptera frugiperda* cells supported this view (Kuroda *et al*., 1990; Butters *et al*., 1991; Wathen *et al*., 1991). In one of these studies (Kuroda *et al*., 1990), it was shown that the influenza virus hemagglutinin contained oligomannosidic side chains, predominantly of the structures $Man_{5-9}GlcNAc_2$, and the truncated oligosaccharide cores Man_3GlcNA_2 and $Man_3[Fluc]GlcNA_2$. An attempt has also been made in this study to allocate these oligosaccharides to specific glycosylation sites on the hemagglutinin. It was already known that hemagglutinin synthesized in chicken cells contained a unique set of oligosaccharides at each attachment site (Keil *et al*., 1985). Of the 5 glycosylation sites of HA_1, Asn 12 contained predominantly triantennary and intersected biantennary structures of the complex type which were highly fucosylated (Figure 1 a). Asn 28 contained oligomnannosidic and biantennary complex structures that lacked fucose. Asn 123 showed biantennary complex structures that were highly fucosylated and sulfated. Asn 149 had biantennary structures with and without intersecting N-acetylglucosamine. Asn 231 had also these structures, but in sulfated form. Of the 2 glycosylation sites of HA_2, Asn 406 had only oligomannosidic side chains, whereas Asn 478 had biantennary complex structures with and without intersecting N-acetylglucosamine. By analogy with these data and based on the fair assumption that complex side chains are replaced by truncated ones, the oligosaccharide structures observed in hemagglutinin derived from SF9 cells could be tentatively allocated to the individual glycosylation sites. As shown in Figure 1b, HA_1 has truncated oligosaccharides at positions 12, 123, 149, and 231, and a mixed set of truncated and oligomannosidic oligosaccharides at position 28. This concept was supported by the observation that most of the oligosaccharides on HA_1 were sensitive to endoglucosaminidaseD and resistant to endoglucosaminidaseH. Furthermore, to total molecular weight calculated for a set of 4.5 truncated oligosaccharides of the average composition $Man_3[Fuc_{0.5}]GlcNAc_2$ (MW c. 1,000 daltons) and 0.5 oligomannosidic side chains of the average composition $Man_7GlcNAc_2$ (MW c. 1,600 daltons) amounts to 5,300 daltons which agree well with the 5,800 daltons derived from the differential electrophoretic mobility of HA_1 in chick embryo and in SF9 cells (Kuroda *et al*., 1989). As also indicated in Figure 1b, the oligomannosidic side chain of HA_2 had most likely to be allocated to Asn 406, since this type of oligosaccharide was found in the same position on hemagglutinin

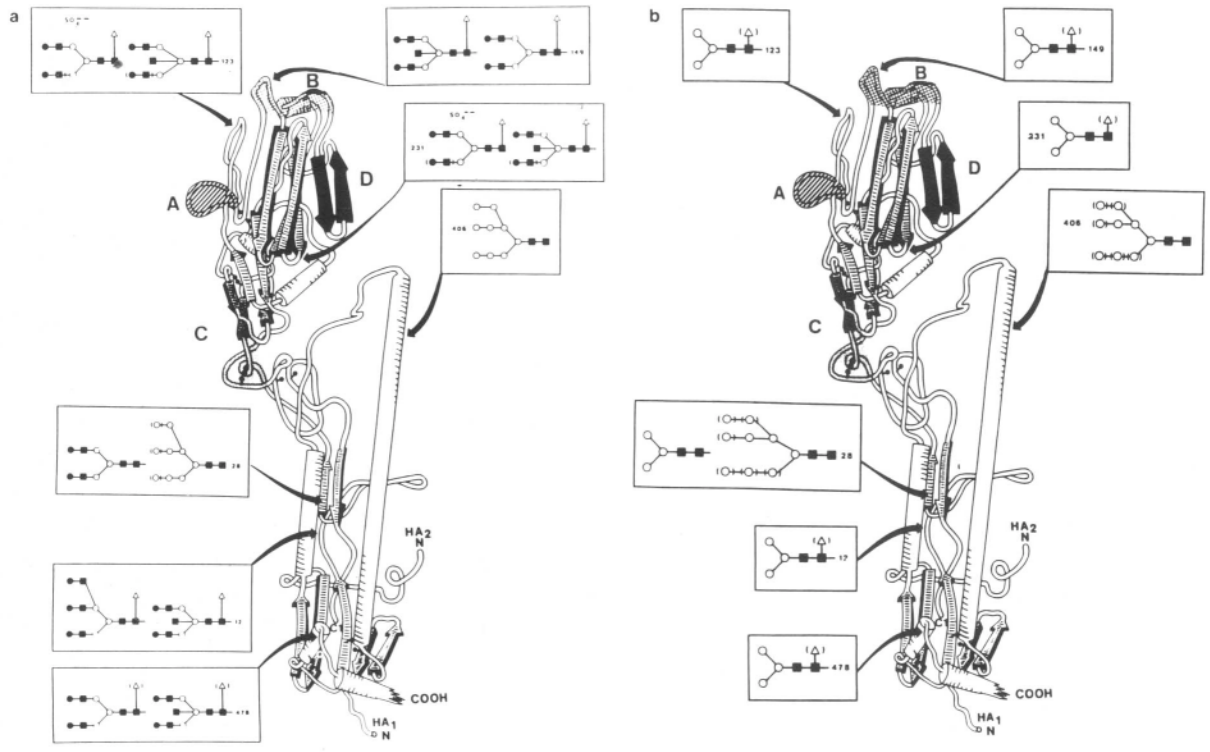

Figure 1. Comparison of the oligosaccharides patterns of hemagglutinin derived from vertebrate and insect cells. The three-dimensional model has been established for the hemagglutinin of strain A/Aichi/68 (H3N2) (Wilson *et al.*, 1981). Left panel: Glycosylation sites and structures of the individual carbohydrate side chains as determined for the hemagglutinin of A/FPV/Rostock/34 (H7N1) grown in chick embryo cells (Keil *et al.*, 1985). Right panel: oligosaccharide structures at individual glycosylation sites of FPV hemagglutinin derived from SF9 cells (Kuroda *et al.*, 1990). ■ N-Acetylglucosamine; ○ mannose; ● galactose; △ fucose. Sequence positions of asparagine residues serving as carbohydrate attachment sites are numbered. Shaded areas indicate antigenic sites A, B, C and D.

from chicken cells. The truncated core oligosaccharide could therefore be attributed to Asn 478. Again, the total molecular weight of 2,600 daltons calculated for these 2 oligosaccharides compared well with the 2,300 daltons estimated from the electrophoretic mobility of HA_2 (Kuroda *et al.*, 1989). These results indicated that, as is the case in vertebrate cells, carbohydrate is attached in SF9 cells to each glycosylation site of the hemagglutinin and they suggested that oligomannosidic side chains are located in positions with restricted accessibility to trimming enzymes. On the other hand, attachment sites furnished with complex oligosaccharides in vertebrate cells contain only truncated side chains when glycosylated in insect cells.

The observation that hemagglutinin synthesized in insect cells has atypical carbohydrates has some interesting implications. From the crystallographic data available (Wilson *et al.*, 1981) it can be estimated that about one third of the total surface of the hemagglutinin spike obtained from chicken cells is covered with carbohydrate. Based on the information available on the spatial conformation of oligosaccharides (Montreuil, 1984), it is fair to assume that the carbohydrate covered area is reduced in hemagglutinin derived from insects by about 50%. This reduction in carbohydrate content may have consequences for the structure and the function of the hemagglutinin. Carbohydrate-protein interactions have been found to be involved in the interface between hemagglutinin subunits and in inter- and intrachain contact regions and have therefore been suspected to stabilize the monomeric and the trimeric structure (Wilson *et al.*, 1981). On the other hand, a supernumerary oligosaccharide has been found to interfere with trimer formation (Schuy *et al.*, 1986). Hemagglutinin with truncated oligosaccharides may therefore display an altered stability. The concept that complete glycosylation stabilizes the trimeric form is supported by the observation that the half time of trimerization is 3 times longer in insect cells than in vertebrate cells. Carbohydrate side chains have also been found to interfere

with receptor binding (Robertson *et al*., 1985; Deom *et al*., 1986) and to shield antigenic epitopes of the hemagglutinin (Skehel *et al*., 1984). Modulation of antibody binding by truncated oligosaccharides was of particular interest, because the FPV hemagglutinin has carbohydrate attached to Asn 149 located in antigen site B (Figure 1). It could be shown, however, that expression in *Spodopterafrugiperda* cells had no effect on the accessibility of this epitope (Munk *et al*., 1992).

It has been reported that human plasminogen contained bi-, tri-, and tetra-antennary complex oligosaccharides with terminal neuraminic acid, when expressed by a baculovirus vector in lepidopteran cells (Davidson *et al*; Davidson & Castellino, 1991 a; Davidson & Castellino, 1991b), and that infection with the baculovirus recombinant enhanced the activity of the trimming enzyme α-D-mannosidase and, thus, controlled the processing of oligomannosidic to complex side chains in these cells (Davidsson *et al*., 1991). It was not clear from these studies, however, whether the control of carbohydrate processing depended specifically on the expression of plasminogen or on infection with the baculovirus vector in general. Although our results obtained on influenza virus hemagglutinin did not support the latter possibility, we considered authentic glycosylation of a recombinant glycoprotein in insect cells an issue important enough for further analysis. We have therefore set out to explore the carbohydrate processing capacity of SF9 cells, the insect cell line used most frequently with baculovirus vectors. The results obtained indicated that SF9 cell do not contain significant amounts of complex N-glycans and that infection with AcMNPV does not induce synthesis of these oligosaccharides. (Kretzschmar *et al*., 1994). These observations are in line with a report by Altmann and coworkers (Altmann *et al*., 1993). Although detecting N-acetylglucosaminyltransferases I and II, both key enzymes in oligosaccharide elongation, these authors found also only minute amounts of complex N-glycans in a number of lepidopteran cells, regardless as to whether they were infected or not. The presence of complex oligosaccharides on human plasminogen derived from SF21 cells (Davidson *et al*., 1990; Davidson & Castellino, 199la; Davidson & Castellino, 1991b) may still be compatible with the view that such glycans appear only after expression of specific proteins. However, other recombinant proteins, such as the influenza virus hemagglutinin (Kuroda *et al*., 1991), the HIV glycoprotein (Buttters *et al*., 1991), and the RSV glycoproteins (Wathen *et al*., 1991) do not show this effect.

Note added in proof: Recent studies have shown that N glycosylation follows in insect cell lines the classical pathway up to the stage of $GlcNacMan_3$-$GlcNAc_2$ oligosaccharide side chains. Whereas these structures are the end product in some cells, they are degraded in others, such as SF9 cells, to $Man_3GlCNac_2$ cores by N-acetyl-β-glucosaminidase.

Altmann F, Schwihla H, Standacher E, Glössl J & März L (1995) Insect cells contain an unusual, membrane-sound β-N-acetylglucosaminidase probably involved in the processing of N-glycans. J. Biol. Chem. 270, 17344–17349.

Wagner R, Geyer H, Geyer R & Klenk H-D (1996) N-Acetyl-β-Glucosaminidase accounts for differences in glycosylation of influenza virus hemagglutinin expressed in insect cells from a baculovirus vector. J. Virol. 70, 4103–4109.

References

Altmann F, Kornfeld G, Dalik T, Staudacher E & Glössl I (1993) Processing of asparagine-linked oligosaccharides in insect cells. N-Acetylglucosaminyltransferase I and II activities in cultured lepidopteran cells. Glycobiology 3, 619–625.

Butters TD, Hughes, RC & Vischer P (1981) Steps in the biosynthesis of mosquito cell membrane glycoproteins and the effects of tunicamycin. Biochim. Biophys. Acta 640, 672–686.

Butters TD, Jones I, Clarke VA & Jacob GS (1991) The effect of glycosidase inhibition on the N-glycans of HIV gp 120 expressed in lepidopteran cells. Glycoconj. J. 8 240.

Davidson DJ & Castellino FJ (1991a) Asparagine-linked oligosaccharide processing in lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells. Biochemistry 30, 6167–6174.

Davidson DJ & Castellino FJ (1991b) Structures of the asparagine-289-linked oligosaccharide assembled on recombinant human plasminogen expressed in a *Mammestra brassicae* cell line (IZD-MBO 503). Biochemistry 30, 6689–6696.

Davidson DJ, Bretthauer RK & Castellino FJ (1991) α-Mannosidase-catalized trimming of high-mannosidase glycans in noninfected and baculovirus-infected *Spodoptera frugiperda* cells (IPBL-SF-21AE). A possible contributing regulatory mechanism for assembly of complextype oligosaccharides in infected cells, Biochemistry 30, 9811–9815.

Davidson DJ, Fraser MJ & and Castellino FJ (1990) Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran insect *(Spodoptera frugiperda)* cells. Biochemistry 29, 5584–5590.

Davies A & Morgan BP (1993) Expression of the glycosylphosphatidylinositol-linked complement-inhibiting protein CD59 antigen in insect cells using a baculovirus vector. Biochem. J. 295, 889–896.

Deom CM, Caton AJ & Schulze IT (1986) Host cell mediated selection of a mutant influenza A virus that has lost a complex oligosaccharide from the tip of the hemagglutinin. Proc. Natl. Acad. Sci. USA 83 3771–3775.

Elbein AD (1987) Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Annu. Rev. Biochemistry 56, 497–534.

Funke C, Becker S, Dartsch H, Klenk H-D & Mühlberger E (1995) Acylation of the Marburg Virus glycoprotein. Virology 208, 289–297.

Hsieh P & Robbins PW (1984) Regulation of asparagine-linked oligosaccharide processing. Oligosaccharide processing in Aedes albopictus mosquito cells. J. Biol. Chem. 259, 2375–2382.

Hu S-L, Kosowski SG & Schaaf KF (1987) Expression of envelope glycoproteins of human immunodeficiency virus by an insect virus vector. J. Virol. 61, 3617–3620.

Keil W, Geyer R, Dabrowski J, Dabrowski U, Niemann H, Stirm S & Klenk J-D (1985) Carbohydrates of influenza virus. Structural elucidation of the individual glycans of the FPV hemagglutinin by two-dimensional ^{1}H NMR and methylation analysis. EMBO J. 4, 2711–2720.

Klenk H-D & Garten W (1995) Activation cleavage in viral spike proteins by host proteases. In: Cellular Receptors for Animal Viruses (Winner E, ed.) pp. 241-279. Cold Spring Harbor Laboratory Press.

Klenk HD & Rott R (1988) The molecular biology of influenza virus pathogenicity. Adv. Virus Res. 34, 247–281.

Kornfeld R & Kornfeld S (1985) Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 54, 631–664.

Kretzschmar E, Geyer R & Klenk H-D (1994) Baculovirus infection does not alter *N*-glycosylation in *Spodoptera frugiperda* cells. Biol. Chem. Hoppe-Seyler. 375, 323–327.

Kuroda K, Geyer H, Geyer R, Doerfler W & Klenk H-D (1990) The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. Virology 174, 418–429.

Kuroda K, Gröner A, Frese K, Drenckhahn D, Hauser C, Rott R, Doerfler W & Klenk H-D (1989) Synthesis of biologically active influenza virus hemagglutinin in insect larvae. J. Virol. 63, 1677–1685.

Kuroda K, Hauser C, Rott R, Klenk H-D & Doerfler W (1986) Expression of the influenza virus haemagglutinin in insect cells by a baculovirus vector. EMBO J. 5, 1359–1365.

Kuroda K, Veit M & Klenk H-D (1991) Retarded processing of influenza virus hemagglutinin in insect cells. Virology 180, 159–165.

Linder ME, Middleton P, Hepler JR, Taussig R, Gilman AG & Mumby SM (1993) Lipid modifications of G proteins: alpha subunits are palmitoylated. Proc. Natl. Acad. Sci. USA 90, 3675–3679.

Montreuil J (1984) Spatial conformation of glycans and glycoproteins. Biol. Cell 51, 115–132.

Munk K, Pritzer E, Kretzschmar E, Gutte B, Garten W & Klenk H-D (1992) Carbohydrate masking of an antigenic epitope of influenza virus haemagglutinin independent of oligosaccharide size. Glycobiology 2, 233–240.

Nayak DP & Jabbar MA (1989) Structural domains and organizational conformation involved in the sorting and transport of influenza virus transmembrane proteins. Annu. Rev. Microbiol. 43, 465–501.

Oker-Blom C, Pettersson RF & Summers MD (1989) Baculovirus polyhedrin promotor directed expression of *Rubella* virus envelope glycoproteins, El and E2, in *Spodoptera frugiperda* cells. Virology 172, 82–91.

Page MJ, Hall A, Rhodes S, Skinner RH, Murphy V, Sydenham M & Lowe PN (1989) Expression and characterization of the Ha-*ras* p21 protein produced at high levels in the insect/baculovirus system. J. Biol. Chem. 264, 19147–19154.

Paulson JC (1989) Glycoproteins: what are the sugar chains for? TIBS 14, 272–276.

Rademacher TW, Parekh RB & Dwek RA (1988) Glycobiology. Annu. Rev. Biochem. 57. 785–838.

Ray R, Galinski MS & Compans RW (1989) Expression of the fusion glycoprotein of human parainfluenza type 3 virus in insect cells by a recombinant beculovirus and analysis of its immunogenic property. Virus Res. 12, 169–180.

Roberts TE & Faulkner P (1989) Fatty acid acylation of the 67K envelope glycoprotein of a baculovirus: *Autographa californica* nuclear polyhedrosis virus. Virology 172, 377–381.

Robertson JS, Naeve CW, Webster RG, Bootman JS, Newman R & Schild GC (1985) Alterations in the hemagglutinin associated with adaptation of influenza B virus to growth in eggs. Virology 143, 166–174.

Roebroek AJM, Creemers JWM, Pauli IGL, Bogaert T & Van de Ven WJM (1993) Generation of structural and functional diversity in furin-like proteins in *Drosophila melanogaster* by alternative splicing of the *DFUR1* gene. EMBO J. 12, 1853–1870.

Roebroek AJM, Creemers JWM, Pauli, IGL, Kurzik-Dumke U, Rentrop M, Gateff EAF, Leunissen JAM & Van de Ven WJM (1992) Cloning and functional expression of Dfurin2, subtilisin-like proprotein processing enzyme of *Drosophila melanogaster* with multiple repeats of a cysteine motif. J. Biol. Chem. 267, 17208–17215.

Schuy W, Will C, Kuroda K, Scholtissek C, Garten W & Klenk H-D (1986) Mutations blocking the transport of the influenza virus hemagglutinin between the rough endoplasmic reticulum and the Golgi apparatus EMBO J. 5, 2831–2836.

Skehel JJ, Stevens DJ, Daniels RS, Douglas AR, Knossow DM, Wilson IA & Wiley DC (1984) A carbohydrate side chain on hemagglutinins of Hongkong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. USA 81, 1779–1783.

Stieneke-Gröber A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk H-D & Garten W (1992) Influenza virus gemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBOJ. 11, 2407–2414.

Vialard J, Lalumière M, Vernet T, Briedis D, Alkhatib G, Henning D, Levin D & Richardson C (1990) Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the β-galactosidase gene. J. Virol. 64, 37–50.

Wathen MW, Aeed PA & Elhammer AP (1991) Characterization of oligosaccharide structures on a chimeric respiratory syncytial virus protein expressed in insect cell line Sf9. Biochemistry 30, 2863–2868.

Webster RG, Laver WG & Air GM (1983) Antigene variation among type A influenza viruses. In: Genetics of Influenza Viruses Palese P & Kingsbury W (Eds) pp. 127–168, Springer Verlag, Wien.

Wiley DC & Skehel JJ (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Ann. Rev. Biochem. 56, 365–394.

Wilson JA, Skehel JJ & Wiley DC (1981) Structure of the hemagglutinin membrane glycoprotein of influenza virus at 3A resolution. Nature (London) 289, 366–373.

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Short Communication

Productivity of insect cells for recombinant proteins

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Key words: insect cell-lines, recombinant proteins, production

The cell line that baculoviruses are grown in ultimately determines how much protein is produced. Insect cell lines have been derived from a variety of insects as well as from different tissues. These cell types include embryonic tissues from developing eggs, ovarian and fat-bodies from immature larvae. These cells have been cultured attached to tissue culture flasks and in suspension cultures in shaker flasks or spinner flasks (Maiorella *et al.*, 1988; Neuka *et al.*, 1992). The passage number from initiation of the culture is an important factor in determining the cells ability to produce virus and recombinant protein. Studies have shown that the cell line is also important in the selection of recombinant baculoviruses by plaque assays (Maruniak *et al.*, 1994). For instance, the number of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) recombinant plaques is higher in *Trichoplusia ni* TN–5B1–4 and TN–368 cells than in the *Spodoptera* cells Sf9 and Sf–21AE.

The ability of a baculovirus to replicate to high titers in a given cell line will determine expression levels of recombinant proteins. Therefore, there is an optimization between which expression vector is chosen and which cell line is used for protein production. Some transfer vectors have been tested in a good number of cell lines while other vectors could be evaluated further. In addition, there are baculoviruses whose potential for high levels of expression have not even been determined. A good example would be the single embedded nuclear polyhedrosis viruses.

The baculovirus expression of recombinant proteins has been tested in quite a few cell lines (Table 1). Factors that were analyzed for these experiments included levels of protein 1) expression 2) glycosylation, 3) phosphorylation, 4) acylation, 5) antigenicity, 6) enzymatic activity, 7) solubility and 8) secretion. One rationale for testing additional cell lines for expression capabilities is that researchers have found that the levels of expression of the foreign genes are consistently lower than the corresponding wild gene whether it be polyhedrin or p10 protein (Luckow & Summers, 1988b;Chaabihi *et al.*, 1992).

The cell lines used in early baculovirus expression experiments were the Spodoptera frugiperda cell lines IPLB-Sf–21 AE and Sf9. Subsequent studies by Davis *et al.*, 1993, showed that cell lines had a significant effect on the ability of baculoviruses to express foreign proteins. They tested eight lepidopteran cell lines with an AcMNPV expression vector. The cell lines tested were compared on the basis of cell density per ml and protein expressed per cell due to the differences in cell size and optimal expression density. An additional factor considered in expression levels was the presence of serum in the media or serum-less medium. The authors used the AcYM1 recombinant expressing secreted alkaline phosphatase (SEAP) under the control of the polyhedrin promoter. In complete TNM-FH medium with 10% fetal bovine serum, BTI–TN–5B1–4 cells produced about ten fold higher levels of SEAP than the next cell line BTI-EA–88 and about 40 times of the least efficient cell line BTI-TN-AP2. The Sf9 and Sf–21AE cells which are still commonly used in baculovirus expression were 20 fold less efficient at expressing the foreign gene.

The most efficient cell line at secreting the recombinant SEAP was MB0503 from Mamestra brassicae

Table 1. Cell lines used for baculovirus expression vectors[a]

Insect	Cell line
Anticarsia gemmatalis	UFL-AG-286
Bombyx mori	BmN[b]
Choristoneura fumiferana	IPRL-Cf-1
Estigmene acrea	BTI-EAA
Heliothis virescens	IPLB-HvT1
Helicoverpa zea	BCIRL-HZ-AM1
Lymantria dispar	IPLB-Ld-EIta
Lymantria dispar	IPLB-LdEItf
Lymantria dispar	IPLB-Ld-652Y[b]
Mamestra brassicae	IZD-MB0503
Mamestra brassicae	NIAS-MaBr-1
Mamestra brassicae	NIAS-MB-25
Mamestra brassicae	SES-MaBr-1
Mamestra brassicae	SES-MaBr-3
Mamestra brassicae	SES-MaBr-4
Manduca sexta	CM-1
Plutella xylostella	BCIRL-PX2-HNV3
Spodoptera exigua	UCR-Se-1
Spodoptera exigua	UCR-Se-1a
Spodoptera frugiperda	IPLB-Sf-21AE
Spodoptera frugiperda	IPLB-Sf-21AE-15
Spodoptera frugiperda	Sf-9
Spodoptera frugiperda	IPLB-Sf-1254
Trichoplusia ni	IPLB-TN-R[2]
Trichoplusia ni	TN-368
Trichoplusia ni	BTI-Tn-5B1-4[b]

[a] From Hink *et al.*, 1991.
[b] Cited in text.

at 85% while the Sf9 was 59% and Sf-21AE 74% of total protein produced. BTI-TN-5B1-4 cells secreted 77% of the expressed protein. When the cell lines were adapted to serum-free EX-CELL 400 medium, the infected cells expressed nearly the same amount of protein. The infected BTI-TN-5B1-4 cells still expressed and secreted (84%) the most protein, while Sf9 expressed the least protein and secreted only 68% (Davis *et al.*, 1993).

Wickham *et al.* (1992), studied these same eight cell lines for the expression of a non-secreted protein, β-galactosidase. The cells producing the most β-galactosidase per million cells were MB0503, 1155 IU followed by the TN-5B1-4 at 1090 IU per million cells. Sf9 and Sf-21AE produced the least, 156 and 177 IU per million cells. The most important factor in the optimal production from the individual cell lines was the density of cells. Reductions in expression were as great as six fold for most cell lines, MB0503 being the most sensitive, while the Sf9 and Sf-21AE were least affected by cell density showing a two fold reduction. Since TN cells are larger than Sf cells, on a per microgram of cell protein basis, TN-5B1-4 and TnM still produced about five fold higher levels of expressed protein.

Hink *et al.* (1991), screened 23 cell lines from 12 different species of Lepidoptera for the expression of three recombinant proteins by baculoviruses. Factors that were considered that could affect yields included whether cell lines were parental or clones, media components and different expression vectors containing foreign genes.

In those studies pAc373 vector was used to construct a baculovirus recombinant expressing pseudorabies gp50, the pAV6 vector with human plasminogen (HPg) and the pAc360 vector was used with β-galactosidase. The cell line producing the most Hpg, 2.6 μg per ml, was the MB0503. It produced three fold higher levels than Sf9, 0.8 μg per ml. Other cell lines from *Spodopterafrugiperda* produced 0.3 to 1.8 μg per ml. *Trichoplusia ni* cells TN-R[2] and TN-368 produced 2.1 and 1.4 μg per ml, respectively.

The expression of β-galactosidase again showed MB0503 cells expressed very high levels, but Sf9 produced slightly higher amounts at 72 h. Several other cell lines produced significantly elevated levels at 48 h, SE-1 and PX2-HNV3, than the other cells. This indicates that not only should cell lines be tested for amounts of protein produced, but also how quickly expression or secretion occurs (Hink *et al.*, 1991).

A different pattern was seen when the pseudorabies gp50 was expressed by the latter authors. The cell lines with the highest relative amount of foreign protein expressed were HvT1 (*Heliothis virescens*), Sf-21AE and Sf-1254. Even though the protein expression was very high, interestingly, the Sf-21 AE showed only 4% visual infection by wild type polyhedra late in infection, while the other cells appeared 40 to 90% infected. Some cell lines, HvT1, LdEIta and Sf-1254, expressed a protein 1000 daltons greater than gp50 in Sf9 cells. This indicates that different cell lines process the carbohydrate moieties differently on glycoproteins. A detailed discussion on glycosylation and other post-translational modifications is presented in another chapter.

There appears to be no consistent rule whether an infected cell line will express one protein better than another in serum containing media or serum free media. For example, Sf-21 AE cells express three fold less β-galactosidase in medium with serum than serum

free medium. The medium composition plays a significant role on how the cell processes affect the expressed gene at either the transcriptional or translational level (Hink *et al.*, 1991).

It is recommended that a number of aspects of the cell lines be considered such as cell growth rate, densities and adaptation to suspension cultures. Due to the cost of serum, alternate cells can be chosen with little effect on levels of protein expressed. A crucial factor is to optimize the expression vector construct, recombinant gene and cell line to maximize production levels.

The levels of expression of the reporter protein β-galactosidase in Sf9 and Sf–21AE compared to TN-368 and MB0503 cells were reported in studies by Wang *et al.* (1992), and King *et al.* (1990). They found approximately the same levels of expressed protein from Sf9 and Sf–21AE cell lines in serum-containing medium which was twice that from TN-368 cells. In contrast, baculovirus expression in either Sf9 or Sf–21AE grown in serum-free media were different. While Sf9 expressed levels comparable to serum supplemented medium, Sf–21AE produced about 20% of the enzyme. King *et al.* (1992), confirmed this in the Sf cells by expressing urokinase at high levels in Sf9 but not in Sf–21AE cells in serumless medium. Meanwhile, MB0503 cells expressed twice as much β-galactosidase as Sf–21 AE cells in serum-containing medium, but urokinase was at the same level in both cells.

Additional baculoviruses have been engineered to express recombinant genes in insect cells. The *Lymantria dispar* (gypsy moth) baculovirus has been engineered to express β-galactosidase under control of the polyhedrin promoter in the *L. dispar* cell line, LD–652Y (Yu *et al.*, 1992). The level of expression reached by recombinant infected cells was 158 IU per million cells at 5 days post infection.

Miyajima *et al.* (1987), used a *Bombyx mori* baculovirus vector to express mouse interleukin-3 in BmN (silkworm) cells. They expressed one million units per ml of culture medium by 3 days. This was 20 times greater than expression of the same gene in COS7 monkey cells by an SV40 expression vector.

An additional study compared the expression of recombinant genes in insect cells, mammalian cells and yeast cells (Tomlinson *et al.*, 1993). The COS7 monkey cells expressed 111 ng per ml of human complement protein C9. The AcMNPV expression vector produced 700 ng per ml in Sf–21 AE cells, while the yeast under the control of several promoters secreted only a very small amount, 5–10 ng per ml medium. The yeast derived C9 was not active, while the mammalian and insect derived complement were fully active in red blood cell lysis experiments.

Many insect cell lines have been tested for the ability to be infected by baculovirus expression vectors. The levels of expression of the recombinant foreign gene have been tested, and it was found that although some cell lines were superior at producing some gene products, other genes were not produced at such levels. Some cells that expressed well in serum containing medium did not adequately express in serum-free medium. Medium did have a strong effect on expression, and it should be optimized for each cell line and each virus. The baculovirus expression system has demonstrated that insect cell lines are more productive than other expression systems that exhibit post-translational modifications of proteins such as glycosylation and phosphorylation. The insect cells secrete biologically active proteins useful in medical and scientific disciplines (Luckow & Summers, 1988). Each cell line that is used in baculovirus expression systems will have to be optimized for scale up experiments that are presented elsewhere in this book. Factors affecting growth rate such as pH, oxygen levels and cells densiites will ultimately determine the quantity of protein expressed.

References

Chaabihi H, Ogliastro M-H, Devachelle G & Cerutti (1992) AcMNPV expression vectors: Comparison between polyhedrin and P10 very late promoters. In Vlak JK, Schlaeger E-J & Bernard AR (eds.). Baculovirus and recombinant protein production processes (pp. 51–55) F Hoffmann La Roche Ltd, Basel.

Davis TR, Wickham TJ, McKenna KA, Granados RR, Shuler ML & Wood HA (1993) Comparative recombinant protein production of eight insect cell lines. In. Vitro. Cell. Dev. Biol. 29A: 388–390.

Hink WF, Thomsen DR, Davidson DJ, Meyer AL & Castellino FJ (1991) Expression of three recombinant proteins using baculovirus vectors in 23 insect cell lines. Biotechnol. Prog. 7: 9–14.

King LA, Mann SG, Marlow SA, Bermudex I, Lawrie AM, Obos LA & Atkinson AE (1992) Alternative insect cell lines for baculovirus expression vectors. In Vlak JM, Schlaeger E-J & Bernard AR (eds.). Baculovirus recombinant protein production processes (pp. 157–165) F Hoffmann La Roche Ltd, Basel.

King LA, Mann SG, Lawrie AM, Mulshaw SH (1991) Replication of wild type and recombinant *Autographa californica* nuclear polyhedrosis virus in a cell line derived from *Mamestra brassicae.* Virus Research 19: 93–104.

Luckow VA & Summers MD (1988a) Trends in the development of baculovirus expression vectors. Biotechnology 6: 47–55.

Luckow VA & Summers MD (1988b) Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology 167: 56–71.

Maiorella B, Inlow D, Shanger A & Harano D (1988) Large scale insect cell-culture for recombinant protein production. Biotechnol. 6: 1406–1410.

Maruniak JE, Garcia-Canedo A & Rodrigues JJ (1994) Cell lines used for the selection of recombinant baculovirus. In. Vitro. Cell. Dev. Biol. Anim. 30A: 283–286.

Miyajima A, Schreus J, Otsu K, Kondo A, Arai K & Maeda S (1987) Use of the silkworm, *Bombyx mori* and an insect baculovirus vector for high-level expression and secretion of biological active mouse interleukin-3 Gene 58: 273–281.

Neuka R, Levi B-Z & Shoham Y (1992) Optimization of protein-production by the baculovirus expression vector system in shake flasks. Appl. Microbiol. Biotechnol. 37: 74–78.

Tomlinson S, Ueda E, Maruniak JE, Garcia-Canedo A, Bjes ES & Esser AF (1993) The expression of hemolytically active human complement protein C9 in mammalian, insect, and yeast cells. Protein. Expr. Purif. 4: 141–148.

Wang, P, Granados, RR & Shuler M (1992) Studies on serum-free culture of insect cells for virus propagation and recombinant protein production. J. Invertebr. Pathol. 59:46–53.

Wickham TJ, Davis T, Granados RR, Shuler ML & Wood HA (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol. Prog. 8: 391–396.

Yu Z, Podgwaite JD & Wood HA (1992) Genetic engineering of a *Lymantria dispar* nuclear polyhedrosis virus for expression of foreign genes. J. Gen. Virol. 73: 1509–1514.

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Chaperone and foldase coexpression in the baculovirus-insect cell expression system

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Abbreviations: BEVS – Baculovirus expression vector system, BiP – immunoglobulin heavy chain binding protein, ELISA – Enzyme-linked immunosorbent assay, ER – Endoplasmic reticulum, GRP – Glucose regulated protein, Hsp – Heat shock protein, IgG – Immunoglobulin G, PDI – Protein Disulfide Isomerase, PPI – Peptidyl-prolyl cis-trans isomerase, Sf–9 – Spodoptera frugeperda,

Introduction

The baculovirus expression vector system (BEVS) has become widely utilized for the production of a variety of recombinant proteins in insect cells (Luckow, 1990; Summers & Smith, 1987). The strong polyhedrin and p10 promoters provide for high levels of recombinant gene expression. Furthermore, as eucaryotes, insect cells will perform many post-translational modifications including disulfide bond formation, glycosylation, and oligomer assembly and are capable of secreting functionally active proteins (Luckow, 1993; O'Reilly *et al*., 1992). Unfortunately, the level of secreted proteins produced from BEVS can be 10 to 100 times lower than the amount obtained for non-secreted proteins (Shuler *et al*., 1995) . This represents a limitation to the wider application of this expression system. Often the polypeptides destined for secretion are improperly processed and can accumulate as protein aggregates within the cell. Developing methodologies to reduce the accumulation of improperly processed proteins and increase secretion from BEVS would enhance the applicability of this expression system. This can be accomplished by manipulating the insect cells' capacity to fold, assemble, and process proteins that are destined for secretion or the outer membrane.

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Folding and assembly of newly translated polypeptides in eucaryotes occurs in the endoplasmic reticulum (ER) for those proteins which are destined to be secreted or exposed on the cell surface. Only after the protein has assumed its final conformation in the ER will the properly assembled structure be transported to the Golgi compartment for eventual secretion into the medium. Anfinsen (1973) demonstrated several decades ago that the final protein structure was dictated by the sequence of amino acids that comprise that protein. Recently, however, it has become evident that folding in the ER is assisted by other proteins present within this folding compartment (Gething & Sambrook, 1992; Helenius *et al*., 1992). These folding assistance factors fall into two major categories: chaperones and foldases.

Folding and secretion assistance factors

Chaperones are believed to improve protein folding and assembly by binding to regions of nascent polypeptides that would otherwise form improper associations and aggregate (Figure 1). By preventing these improper associations, the chaperones allow the polypeptide to fold and assemble into the correct conformation. Foldases facilitate protein folding by catalyzing slow steps in folding such as the rearrangement of disulfide bonds or the *cis-trans* isomerization of peptidyl-prolyl bonds (Wittrup, 1995; Fischer, 1994). Chaperones and foldases are present in the ER and other cellular compartments where they serve multiple roles: assisting in folding and assembly, facilitating transport of newly

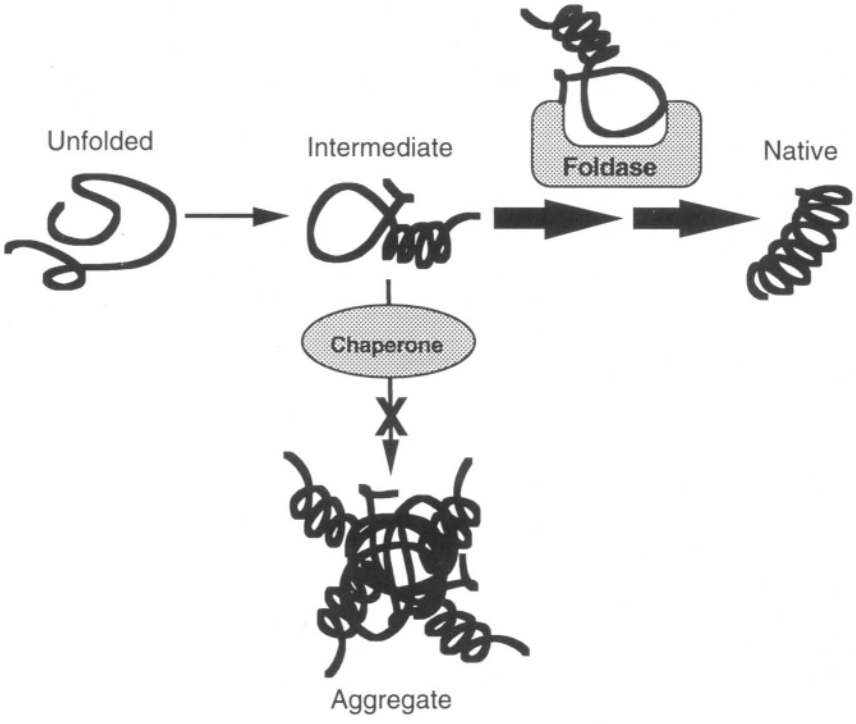

Figure 1. Folding and assembly of proteins can be assisted by chaperones and foldases. Chaperones can prevent aggregation while foldases act as catalysts to facilitate protein folding.

translated polypeptides, and binding to denatured and unfolded proteins (Georgopoulos, 1992). In addition to chaperones and foldases, there are other cellular proteins involved in folding, assembly and secretion. These include signal peptidases, which cleave signal peptides from newly translocated proteins, and glycosidases, which add and modify oligosaccharides attached to amino acids (Rothblatt *et al.*, 1994). Transport of the fully assembled structure from the ER through the Golgi apparatus requires additional cellular proteins. If the levels of one or several of these post-translational factors are insufficient, the secretion of complex proteins from a particular cell line may be reduced.

In a normal cell, the concentration of these catalytic proteins is likely to be sufficient to provide for proper protein folding and assembly of a limited number of proteins destined for secretion. However, in BEVS and other genetically engineered cells, the modified cell may be required to fold, assemble, and secrete many more proteins than the native organism can accommodate. Also, the baculovirus infection can compromise the host cell's capacity to assimilate heterologous proteins (Jarvis & Summers, 1989). As a result, the cell's folding, assembly, and secretion machinery may be saturated leading to the formation of improperly folded structures (abnormal complexes) and protein aggregates.

Protein aggregation

The accumulation of improperly processed proteins and aggregates is a significant problem in many recombinant DNA expression systems for both simple and complex protein structures (Mitraki *et al.*, 1991). Protein aggregates are found in *E. coli* (where they are called "inclusion bodies"), yeasts, mammalian cells, and insect cells (Georgiou & Bowden, 1990; Marquardt & Helenius, 1992). Haseman & Capra (1990) used the baculovirus expression vector for the production and secretion of functionally active immunoglobulin G (IgG) antibodies in insect cells. However, secretion of functional IgG was accompanied by the formation of "incorrectly associated chains" and aggregates (Haseman & Capra, 1990; Hsu *et al.*, 1994b). Furthermore, recent research has indicated that a number of proteins, including human chorionic gonadotropin (hcg) α and β subunits (Nakhai *et al.*, 1991), major histocompatibility complex (MHC) antigens (Stern &

Wiley, 1992), tissue plasminogen activator (Jarvis & Summers, 1989), pre-proattacin (Gunne *et al*., 1990), HIV envelope protein (Murphy *et al*., 1990), insulin receptor (Paul *et al.*, 1990; Sissom & Ellis, 1991), and protein kinase c-δ (Rankl *et al*., 1994), aggregate or are poorly processed and transported in BEVS. The formation of these aggregates suggests that the post-translational processing capacity of BEVS may be unable to accommodate high expression levels of these proteins.

Methods to alleviate protein aggregation

Several approaches have been undertaken to alleviate the improper processing of secreted and membrane proteins that can occur in BEVS. The utilization of the basic (core) promoter is one alternative. The basic promoter provides for lower expression of recombinant proteins earlier in the infection cycle when cellular processing is not yet compromised in the infected insect cells. This approach has resulted in improved secretion levels of functionally active proteins (Sridar & Hasnain, 1993; Rankl *et al*., 1994) but does not take advantage of the strength of the very strong late promoters of polyhedrin and p10. Another method to reduce poor secretion is the use of different signal peptides and sequences. The application of the honeybee melittin signal peptide (Tessier *et al*., 1991) and the baculovirus egt or gp67 signal peptides (Murphy *et al*., 1993) was observed to enhance secretion of some poorly processed proteins. However, the utilization of different signal peptides does not always facilitate enhanced secretion of certain complex proteins such as tissue plasminogen activator (Jarvis *et al*., 1993).

Another approach is to engineer the folding and assembly pathway to include chaperones and foldases in concert with the genes for the secreted proteins. Since the intracellular catalyst concentration may be severely limited in a host cell, one way to overcome this limitation is to increase the intracellular concentration of the catalyst(s). This may be accomplished by cloning the gene for a chaperone or other catalytic protein into BEVS and coexpressing the chaperone/foldase in concert with the genes coding for the final product. These coexpressed proteins can facilitate the proper folding and assembly of the final product protein and prevent aggregation.

There are a number of chaperones and foldases present within the ER. Different chaperones and foldases have been observed to be important for the folding of certain secreted and membrane proteins. Two proteins that play important roles in the folding, assembly, and secretion of numerous proteins from eucaryotic cells are immunoglobulin heavy chain binding protein (BiP) and protein disulfide isomerase (PDI). Given their important roles in protein folding, BiP and PDI are likely candidates to be limited in a recombinant expression system. These two assistance factors will be reviewed in following sections followed by an evaluation of the effects of coexpressing BiP and PDI on the production of heterologous proteins in BEVS. Subsequent sections will explore other chaperones and foldases which may be limited in BEVS and represent future targets for coexpression.

Chaperones and foldases

Immunoglobulin heavy chain binding protein (BiP)

A member of the Hsp70 family of heat shock proteins and chaperones, immunoglobulin heavy chain binding protein (BiP) was first isolated in association with immunoglobulin heavy chains (Haas & Wabl, 1983) and has since been associated with other antibody intermediates (Bole *et al*., 1986). BiP functions by interacting with unfolded polypeptides to prevent polypeptide aggregation or to maintain the chains in a conformation which is readily associated with the other chains during proper assembly (Rothman, 1989). In addition to immunoglobulin heavy chains, BiP has been observed to associate with a number of other polypeptides including immunoglobulin light chains (Knittler, 1992), influenza hemagglutination subunits (Gething & Sambrook, 1989), vesicular stomatitis virus G protein (de Silva *et al*., 1990), thyroglobulin (Kim *et al*., 1992), and the HIV envelope protein (Earl *et al*., 1991). In normal cells, BiP is constitutively expressed and represents approximately 1% of the cellular protein (Pelham, 1989; Rothman, 1989). BiP has been localized to the ER compartment and includes the carboxy terminal amino acid KDEL sequence for retention in the ER. Its synthesis level can be increased under stress conditions such as glucose starvation and when abnormal proteins accumulate in the ER (Lee, 1987; Pelham, 1989). In addition to preventing polypeptide aggregation, BiP may be involved with the translocation of secretory proteins into the ER (Vogel *et al*., 1990).

Recent research in several laboratories has elucidated the general characteristics of BiP binding to nascent polypeptides. The binding site on BiP will

accommodate a linear sequence of seven amino acids and includes four pockets for hydrophobic or aromatic residues (Blond-Elguindi *et al.*, 1993; Flynn *et al.*, 1991). Occupancy of at least two pockets by favored residues is sufficient to facilitate BiP binding. There is a computer program that can be used to predict BiP binding sites in proteins (Blond-Elguindi *et al.*, 1993). These binding sequences, which are likely sites of aggregation, are common in many protein sequences and often will be hidden in the interior of the folded protein.

BiP coexpression in insect cells

Since expression and secretion of active IgG molecules from insect cells is accompanied by the formation of abnormal complexes and aggregates (Haseman & Capra, 1990), it was hypothesized that the level of BiP may be insufficient to facilitate folding and assembly of functional IgG antibodies.

To eliminate the BiP limitation, the gene for murine BiP (Haas & Meo, 1988) was cloned into the baculovirus vector AcBB (Jeang *et al.*, 1987) under the control of the late polyhedrin promoter. In order to show that heterologous BiP was active in insect cells, an association of recombinant BiP with the immunoglobulin chains was proved by immunoprecipitation with protein A (Hsu *et al.*, 1994b). The effect of coexpressing BiP on intracellular and extracellular functional and soluble IgG was evaluated in both High Five *(Trichoplusia ni*, BTI-TN5-B–4) (Davis *et al.*, 1993) and *Spodoptera fruglperda* (Sf–9) insect cells. Soluble IgG was several fold higher in the clarified lysates from infected High Five cells coexpressing BiP at 3 days post-infection. Secreted antibody levels were also observed to increase (Hsu, unpubl.). Similar experiments performed in Sf–9 cells indicated that BiP coexpression increased intracellular antibody levels while antibody levels in the medium did not change significantly (Hsu *et al.*, 1994b). Preliminary experiments in other laboratories have indicated that BiP coexpression in High Five cells can increase secretion of other proteins from the immunoglobulin superfamily (Crowe, pers. comm.).

Subsequent experiments in High Five cells indicated that BiP was increasing the level of soluble labeled nascent immunoglobulins immediately following a pulse of radioactive amino acids (Hsu, unpubl.). Consequently, BiP appears to be functioning as a true chaperone to prevent aggregation of the nascent immunoglobulin chains prior to their assembly into

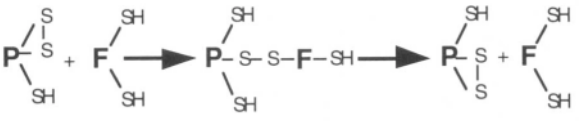

Figure 2. Isomerization of a protein (**P**) intramolecular disulfide bond catalyzed by PDI (**F**) (Freedman *et al.*, 1994).

intermediates and functional antibodies. Future studies will evaluate the role of BiP in preventing aggregation of other secreted proteins that include putative BiP binding sites.

Protein disulfide isomerase (PDI)

Numerous secreted proteins include disulfide bonded crosslinks between cysteine residues. These bonds may serve to enhance the structural stability of the protein in the extracellular environment (Wittrup, 1995). A large amount of evidence now suggests that the formation of these disulfide bonds *in vivo* can be assisted by the cellular catalyst, protein disulfide isomerase (PDI). PDI is a 57 kDa protein found in the ER compartment as a dimer that contains active site thiols which can catalyze the formation, breakage, or isomerization of disulfide bonds for cysteine-containing polypeptides. The isomerization reaction of a polypeptide (**P**) that is catalyzed by PDI (**F**) is shown in Figure 2. PDI shows internal homologies with the small redox protein, thioredoxin, also involved in disulfide interchange reactions (Pigiet & Schuster, 1986).

In vitro studies have shown that PDI will catalyze the formation of native disulfide bonds in a number of proteins including bovine ribonuclease A (Lyles & Gilbert, 1991), bovine trypsin pancreatic inhibitor (Weissman & Kim, 1993), human chorionic gonadotropin (Huth *et al.*, 1993) and murine immunoglobulin $\mathbf{F}_{ab}$ fragments (Lilie *et al.*, 1994). Studies with microsomal vesicles have indicated that PDI can facilitate disulfide bond formation for nascent proteins (Bullied & Freedman, 1988), and *in vivo* evidence has linked PDI to the formation of native disulfide bonds in yeast (Lamantia & Lennarz, 1993). PDI, which also includes a peptide-binding domain, may also possess chaperoning as well as antichaperoning activities (Noiva & Lennarz, 1992; Puig & Gilbert, 1994).

Another protein to which PDI has been indirectly linked in both *in vitro* and *in vivo* studies is IgG, which includes a number of intrachain and interchain disulfide bonds (Figure 3). A close correlation between

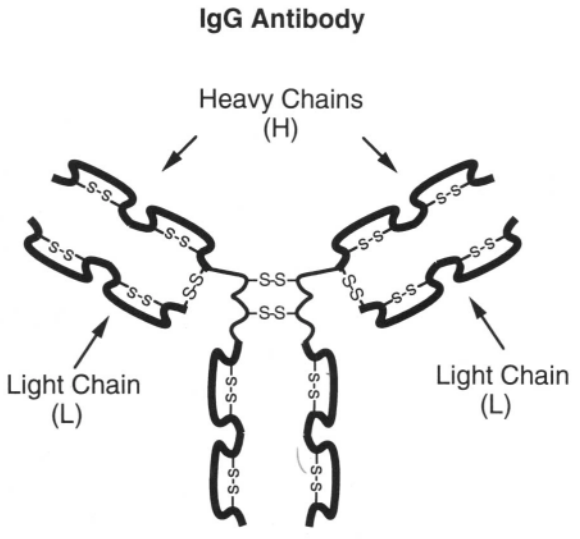

Figure 3. Complete antibody oligomer composed of two heavy (H) chains and two light (L) chains.

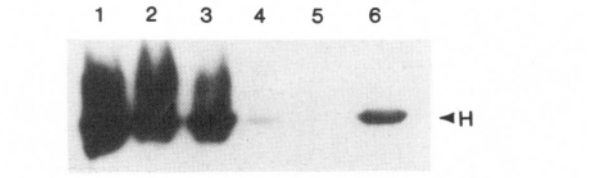

Figure 4. Western blot analysis of recombinant immunoglobulin heavy (H) chain levels in Sf–9 insect cells. Equal amounts of whole cell lysates and clarified cell lysates were analyzed under reducing conditions in each lane. Clarified lysates were obtained from whole cell lysates by centrifugation at 16 000 g for 5 minutes. Whole cell lysates, lanes 1, 2, and 3, are from cells infected with JPLH, JPLH+AcBB, JPLH+AcBB–PDI, respectively. Clarified cell lysates, lanes 4, 5, and 6, are from cells infected with JPLH, JPLH+AcBB, JPLH+AcBB-PDI, respectively.

IgG secretion levels and PDI levels was apparent in a study of B cell maturation (Freedman *et al*., 1989). In addition, a study of a number of lymphocytes and lymphoma derived cells demonstrated a very close correlation between PDI activity levels and IgG synthesis rates (Roth & Koshland, 1981). Furthermore, PDI was observed to crosslink with nascent immunoglobulin chains (Roth & Pierce, 1987).

PDI coexpression in insect cells

Since PDI has been linked to IgG formation, the effect of coexpressing PDI catalyst on murine IgG production was evaluated in BEVS. The gene for mouse PDI (Mazzarella, 1990) was cloned into baculovirus (AcBB-PDI) (Hsu *et al*., 1994a). Sf–9 insect cells were coinfected with this baculovirus and a baculovirus containing the murine IgG gene (JPLH) (zu Pulitz *et al*., 1990). An immunoblot of the intracellular immunoglobulin protein content revealed a significant increase in the soluble IgG heavy chain level for cells coinfected with AcBB-PDI as compared to a coinfection with a control (AcBB) and to an infection with JPLH alone (Figure 4). Levels of functional IgG antibody, as measured by ELISA, also were enhanced in the presence of PDI after 3 days post-infection (Hsu *et al*., 1996).

Pulse-chase labeling experiments were used to elucidate the role of PDI in BEVS. In the presence of PDI, soluble labeled immunoglobulin levels were observed to increase over the entire chase period indicating that PDI was salvaging immunoglobulin chains from aggregates. The capacity for PDI to rescue immunoglobulins would explain the higher IgG secretion levels observed in the cells coexpressing PDI.

In addition to its role as an enzyme, PDI also serves as an integral component of several complex multimeric proteins. PDI comprises the β subunit of the $\alpha_2\beta_2$ tetrameric enzyme prolyl–4-hydroxylase while the α subunits contribute the active site of the enzyme. Expression of the α subunits in insect cells in the absence of PDI resulted in the formation of an inactive and insoluble product; whereas, coinfection of the cells with viruses coding for α and β subunits resulted in an active enzymatic tetramer (Vuori *et al*., 1992a). Interestingly, the active site cysteine residues on PDI were not essential for the solubility and integrity of the native heterotetramer (Vuori *et al*., 1992b). The fact that PDI can salvage immunoglobulins and solubilize prolyl–4-hydroxylase demonstrates the multifunctional character of this enzyme. Since many secreted proteins include disulfide bonds, PDI may be appropriate for increasing secretion of these proteins if they are improperly processed in BEVS.

Other folding assistance factors

In addition to BiP and PDI, several other ER resident proteins, including PPI, GRP94, and calnexin, have been observed to serve chaperoning or folding assistance functions *in vitro* and *in vivo*. These assistance factors may be limited during recombinant gene expression and represent appropriate candidates for coexpression in BEVS. In addition, chaperones and foldases of the ER may act in concert to facilitate protein folding and assembly. Consequently, it may be

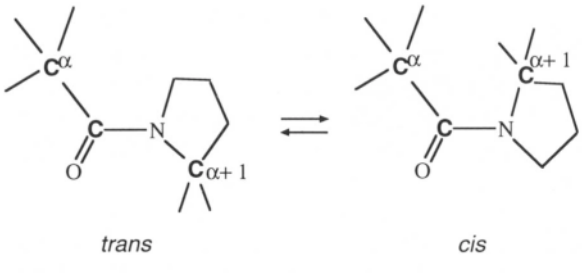

Figure 5. Cis and *trans* isomeric forms of an Xaa-Pro peptide bond.

necessary to coexpress several factors simultaneously to minimize aggregation and maximize secretion of complex proteins. The characteristics of these assistance proteins will be described in following sections.

Peptidyl-prolyl Cis-trans isomerase (PPI)

The peptide Xaa-Xaa bond (where Xaa represents any amino acid) between two amino acids is generally planar and in the *trans* isomeric state. However, the Xaa-Pro peptide bond can exist in either the *cis* or *trans* conformation (Figure 5) and about 7% of these peptide bonds are in the *cis* conformation in native proteins. The proline hypothesis states that many slow transitions in protein folding are the result of slow isomerizations of one or more Xaa-Pro bonds (Brandts *et al.*, 1975). This may be due to the high energy of activation (E_A approx. 20 kcal/mol) required for rotations about the partial double bond of Xaa-Pro peptide bonds. Time constants from 10 to 100 seconds have been reported for the reaction *in vitro* (Cheng & Bovey, 1977; Grathwohl & Wuthrich, 1976a, 1976b, 1981)

PPI, an ubiquitously expressed enzyme in many organisms and subcellular compartments, was first identified by its activity in catalyzing the *cis-trans* isomerization of the short peptide succinyl-Ala-Ala-Pro-Phe–4-nitroanilide (Fischer *et al.*, 1984). Known collectively as immunophilins, two distinct and structurally dissimilar classes of PPIs have been discovered: cyclophilins and FK506-binding proteins. In addition to their PPI activities both are known to bind specific immunosuppressants with high affinity. Cyclophilins bind the immunosuppressant cyclosporin A (CsA) (Handschumacher *et al.*, 1984), whereas FK506-binding proteins bind the immunosuppressants FK506 and rapamycin (Siekierka *et al.*, 1989).

Following the discovery of PPI a number of investigators evaluated PPI as a catalyst for protein folding. Rate enhancement by prolyl isomerization *in vitro* has been demonstrated for the slow folding phases of mouse IgG light chain, porcine RNase (Lang *et al.*, 1987), RNase T1 (Fischer, 1989; Kiefhaber *et al.*, 1990; Schmid, 1993), mouse antibody fragment MAK 33 F_{ab} (Lilie *et al.*, 1993), human carbonic anhydrase II (HCA II) (Fransson *et al.*, 1992; Fresgard *et al.*, 1992), and collagen (Bachinger, 1987; Davis *et al.*, 1989). In the presence of PPI the slow refolding phase of murine IgG light chain was accelerated up to seven fold, depending on the PPI concentration (Lang *et al.*, 1987). Also, PPI was shown to accelerate the rate of refolding of the monoclonal antibody F_{ab} fragment (MAK33 F_{ab})by 1.5 to 3.5 times (Lilie *et al.*, 1993). Addition of the PPI inhibitor cyclosporin A eliminated this rate enhancement.

The effect of PPI on *in vivo* protein folding is not well characterized. Indirect evidence of PPI involvement is suggested by retardation of the *in vivo* maturation of two proteins, procollagen (Steinmann *et al.*, 1991) and transferrin (Lodish & Kong, 1991), in the presence of cyclosporin A. Correct processing of rhodopsin also has been attributed to the presence of the PPIase NinaA (Colley *et al.*, 1991; Stamnes *et al.*, 1991). Knappik *et al.* (1993) reports slight changes in F_{ab} production in *E. coli* in the presence of coexpressed PPI. Also, PPI has been expressed in BEVS (Caroni *et al.*, 1991; Ailor, unpubl.). Given the slow nature of *cis-trans* prolyl isomerizations and the importance of this folding step in many proteins, coexpression of PPI in BEVS has the potential to accelerate folding and enhance production of candidate proteins.

Glucose-regulated protein 94 (GRP94)

The Hsp90 family is another collection of heat shock proteins that are important to protein folding and are expressed at amplified levels under stress conditions (Jakob & Buchner, 1994). Hsp90 is the most abundant cytosolic protein in eucaryotic cells (Jakob & Buchner, 1994) and has a homologue in the endoplasmic reticulum of higher eucaryotes, GRP94, also known as endoplasmin, ERp99, and gp96 (Melnick *et al.*, 1994).

The exact function of GRP94 and other Hsp90 family members is not well understood. Initially, it was believed that the role of the Hsp90 family was to serve a regulatory function, blocking activation by steric interference and stabilizing unfolded proteins until they were properly localized (Craig *et al.*, 1993). Now it is believed that Hsp90 is also required for the functional activation of these proteins and perhaps for their folding into an active conformation. Recent stud-

ies *in vitro* have indicated that the Hsp90 family has general chaperone properties like those of GroE and BiP (Weich *et al*., 1992; Jakob & Buchner, 1994). In the presence of Hsp90, the yield of folded F_{ab} fragments of monoclonal antibodies increased from 40 to 60% (Weich *et al*., 1992). Refolding studies on protein citrate synthase indicated that a two-fold excess of Hsp90 suppressed aggregation to 50% of the available protein while an eight-fold excess suppressed aggregation entirely (Weich *et al*., 1992). Chaperone action is believed to occur through a mechanism of binding and release followed by subsequent rebinding until a native or near-native state is reached (Jakob & Buchner, 1994). While BiP and other Hsp70 proteins appear to bind preferentially to small peptide regions, Hsp90 appears to bind to proteins rather than peptides (Li & Srivastava, 1993) and may represent a later stage chaperone in protein folding.

GRP94 has been linked to proper folding and functional activation of proteins in the ER and can be induced by the accumulation of malfolded proteins (Kozutsumi *et al*., 1988). GRP94 has been found in association with major histocompatibility complex class II polypeptides (MHC II) (Schaiff *et al*., 1992), a mutant herpes glycoprotein (Navarro *et al*., 1991), and immunoglobulins (Melnick *et al*., 1992). Melnick and coworkers (1992) observed that GRP94 interacts with newly synthesized immunoglobulin chains and suggested that this protein, like BiP, was acting as a molecular chaperone. Since BiP bound to early reduced forms of the light chain and GRP94 bound to more mature oxidized forms, the authors suggested that BiP and GRP94 act in concert to stabilize the conformation of free heavy and light chains (Melnick *et al*., 1994). This concerted action supports the hypothesis put forth by Li & Srivistava (1993) that GRP94 and BiP modulate and collaborate with each other to assist in protein folding and assembly. Such chaperone collaborations *in vivo* may be appropriate in baculovirus and other expression systems to reduce aggregation and increase yields of complex proteins. GRP94 has been expressed in BEVS (Whiteley, unpubl.) and its effect on the production of IgG in insect cells is being evaluated.

Calnexin

Calnexin (also known as p88 and IP90) is the first membrane chaperone to be associated with folding and assembly of proteins in the ER. The mature calnexin protein includes a luminal domain that binds calcium, a transmembrane sequence, and a short cytoplasmic tail at the C-terminus (Helenius, 1994; Wada *et al*., 1994). This acidic cytoplasmic tail is believed to be responsible for calnexin's mobility of approximately 90 kDa on SDS-PAGE (Helenius, 1994). Calnexin also shows high sequence similarity with the ER luminal calcium binding protein, calreticulin.

Calnexin was first identified as a chaperone in association with a number of membrane subunits including MHC antigens (Helenius, 1994; Anderson & Cresswell, 1994), influenza trimers (Helenius, 1994), the cystic fibrosis transmembrane conductance regulator (Pind *et al*., 1994), and membrane-bound immunoglobulins (Hochstenbach *et al*., 1992). More recently, calnexin has been found to associate with secreted glycoprotein folding intermediates including transferrin, α1-antitrypsin, α-fetoprotein (Ou *et al*., 1993), gp80 (Wada *et al*., 1994), and thyroglobulin (Kirn & Arvan, 1995) prior to folding and transport from the ER. Prolonged association with calnexin can occur when folding, disulfide bond formation, or oligomeric assembly is restricted (Helenius, 1994; Wada *et al*., 1994). How calnexin binds to intermediates is currently under investigation. Calnexin may bind to selective carbohydrates within glycoproteins in a lectin-like binding fashion (Helenius, 1994; Ou, *et al.* 1993) or it may bind to hydrophobic patches exposed prior to complete intra- and interchain disulfide bond formation (Wada *et al*., 1994). The membrane location of calnexin may allow this chaperone to bind and tether newly synthesized hydrophobic domains in nascent polypeptides. The calnexin structure includes a large hydrophobic helix at residues 402 to 422 that could interact with hydrophobic patches on newly synthesized polypeptides (Wada *et al*., 1994).

Recent evidence has accumulated to indicate that calnexin acts in concert with other ER chaperones. Calnexin and BiP were observed to bind in sequential order to thyroglobulin (Kim & Arvan, 1995) and VSV G (Hammond & Helenius, 1994) proteins prior to their maturation in the ER.

Preliminary evidence in our laboratories (Hinderliter, unpubl.) indicates that calnexin can act as a chaperone in the baculovirus expression system to enhance the folding, assembly, and secretion of eucaryotic proteins. Coexpression of calnexin may be especially advantageous for enhancing the folding and assembly of membrane proteins which are poorly processed and aggregate in BEVS.

Cytosolic chaperones in insect cells

In addition to their presence in the ER, chaperones are found in the cytosol and other cellular compartments. In the cytosol, they can prevent aggregation and facilitate transport of unfolded polypeptides to another compartment. Thus, overexpression of cytosolic chaperones may be important to enhancing intracellular and secreted protein levels from BEVS.

The oligomeric steroid binding receptors, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), have been expressed in BEVS but less than 1% of the protein was active while the remainder was observed to aggregate extensively (Alnemri & Litwack, 1993). Since these receptors are known to interact with the cytosolic heat shock proteins prior to assembly, the Hsp90 and Hsp70 chaperones were coexpressed in BEVS along with the receptors (Alnemri & Litwack, 1993). Coexpression failed to alleviate the aggregation or to increase formation of the active steroid binding receptor complexes, possibly due to the requirement for other cellular factors. However, Hsp90 and Hsp70 in BEVS may be effective for increasing the solubility of recombinant proteins that do not require additional cellular factors for proper folding and assembly.

Other folding, assembly, and secretion factors

Enhancing the folding and assembly environment in the ER may not always be effective for increasing secretion of complex proteins. Other post-translational processing steps including signal peptide processing, oligosaccharide processing, and protein transport may limit BEVS' capacity to produce functional secreted and membrane proteins.

Cellular protein transport is an energy driven process that involves a number of proteins, including coatomer and the ADP-ribosylation factor (ARF) (Rothman, 1994). Since the baculovirus infection alters insect cell physiology (Mitchell-Logean & Murhammer, 1995), this may affect protein transport. Coexpression of one or several of the transport facilitating proteins may improve the movement of assembled proteins through the insect secretory apparatus.

The oligosaccharide processing of a protein may limit secretion (Sissom & Ellis, 1991) or else be used to identify possible limiting steps in secretion. If oligosaccharide processing enzymes are suspected of limiting secretion, genetic engineering approaches can be employed to express the oligosaccharide modifying enzymes. This "glycosylating engineering" (Jenkins & Curling, 1994) approach has been used successfully in mammalian cells (Lee, 1987; Smith *et al*., 1990; Potvin *et al*., 1990). Alternatively, one can alter the glycoforms of the secreted proteins from insect cells by including genes for glycosylating enzymes not present or at low levels in insect cells. Such a "glycosylating engineering" approach would complement current investigations of alternative insect cell lines (Davis *et al*., 1993; Pasumarthy & Murhammer, 1994) and has the potential to be as successful as efforts to modify the ER environment with coexpressed chaperones and foldases.

Conclusions

The BEVS has become widely utilized for production of recombinant proteins. However, protein aggregation and inefficient processing often limit yields, especially for secreted and membrane proteins. Since many proteins of pharmaceutical interest require similar post-translational processing steps, engineering the folding, assembly, and secretion pathway may enhance the production of a wide variety of valuable complex proteins. Efforts should be undertaken to coexpress the relevant chaperones or foldases at low levels in concert with the final product to ensure the ideal folding and assembly environment. In the future, expression of oligosaccharide modifying enzymes and secretion factors may further improve secretion rates of assembled proteins and provide heterologous proteins with altered glycoforms. Also significant is the use of BEVS as an *in vivo* eucaryotic laboratory to study the fundamental roles of different chaperones; foldases, and secretion factors. The coexpression of chaperones and foldases will complement other approaches such as the development of alternative insect cell lines, promoters, and signal peptides to optimize the baculovirus-insect cell expression system for generating high yields of valuable proteins.

References

Alnemri ES & Litwack G (1993) The steroid binding domain influences intracellular solubility of the baculovirus overexpressed glucocorticoid and mineralocorticoid receptors. Biochemistry 32: 5387–5393.

Anderson KS & Cresswell P (1994) A role for calnexin (IP90) in the assembly of class II MHC molecules. EMBO J. 13: 675–682.

Anfinsen CB (1973) Principles that govern the folding of protein chains. Science 181: 223–230.

Bachinger HP (1987) The influence of peptidyl-prolyl cis-trans isomerase on the in vitro folding of type III collagen. J. Biol. Chem. 262: 17144–17148.

Blond-Elguindi S, Fourie AM, Sambrook JF & Gething MJ (1993) Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell 75: 717–728.

Bole DG, Hendershot LM & Kearney JF (1986) Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. 102: 1558–1556.

Brandts JF, Halvorson H & Brennan M (1975) Consideration of the possibility that the slow step in protein denaturation reactions is due to cis-trans isomerism of proline. Biochemistry 14: 4953–4963.

Bulleid NJ & Freedman RB (1988) Defective co-translational formation of disulphide bonds in protein disulphide-isomerase-deficient microsomes. Nature 335: 649–651.

Caroni P, Rothenfluh A, McGlynn E & Schneider C (1991) S-cyclophilin: new member of the cyclophilin family associated with the secretory pathway. J. Biol. Chem. 266: 10739–10742.

Cheng HN & Bovey FA (1977) Cis-trans equilibrium and kinetic studies of acetyl-L-proline and glycyl-L-proline. Biopolymers 16: 1465–1472.

Colley NJ, Baker EK, Stamnes MA & Zuker CS (1991) The cyclophilin homolog ninaA is required in the secretory pathway. Cell 67: 255–263.

Craig EA, Gambil BG & Nelson J (1993) Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. 57: 402–414.

Davis JM, Boswell BA & Bachinger HP (1989) Thermal stability and folding of type IV procollagen and effect of peptidyl-prolyl cis-trans-isomerase on the folding of the triple helix. J. Biol. Chem. 264: 8956–8962.

Davis TR, Shuler ML, Granados RR & Wood HA (1993) Comparison of oligosaccharide processing among various insect cell lines expressing a secreted glycoprotein. In Vitro Cell. Dev. Biol. 29A: 842–846.

de Silva AM, Balch WE & Helenius A (1990) Quality control in the endoplasmic reticulum: folding and misfolding of vesicular stomatitis virus G protein in cells and in vitro. J. Cell Biol. 111: 857–866.

Earl PL, Moss B & Doms RW (1991) Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J. Virology 65: 2047–2055.

Fischer G, Bang H & Mech C (1984) Determination of enzymatic catalysis for the cis-trans- isomerization of peptide binding in proline-containing peptides. Biomed. Biochim. Acta 43: 1101–1111.

Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T & Schmid, FX (1989) Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. Nature 337: 476–478.

Fischer G (1994) Peptidyl-prolyl *cis/trans* isomerases and their effectors. Angew. Chem. Int. Ed. Engl. 33: 1415-1436.

Flynn GC, Pohl J, Flocco MT & Rothman JE (1991) Peptide-binding specificity of the molecular chaperone BiP. Nature 353: 726–730.

Fransson C, Freskgard PO, Herbertsson H & Johansson A (1992) *Cis-trans* isomerization is rate-determining in the reactivation of denatured human carbonic anhydrase II as evidenced by proline isomerase. FEBS Lett. 296: 90–94.

Freedman RB, Bulleid NJ, Hawkins HC & Paver JL (1989) Role of protein disulphide-isomerase in the expression of native proteins. Biochem. Soc. Symp. 55: 167–192.

Freedman RB, Hirst TR & Tuite MF (1994) Protein disulphide isomerase: building bridges in protein folding. Trends Biochem. Sci. 19:331–336.

Fresgard PO, Bergenhem N, Jonsson BH, Svensson M & Carlsson U (1992) Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. Science 258: 466–468.

Georgiou G & Bowden GA (1990) Inclusion body formation and the recovery of aggregated recombinant proteins. In: Ho C, Prokop A & Bajpai R (eds) Recombinant DNA Technology and Applications, (pp. 333–356) McGraw-Hill, New York.

Georgopoulos C (1992) The emergence of the chaperone machines. Trends Biochem Sci. 17: 295–299.

Gething MJ & Sambrook J (1989) Protein folding and intracellular transport: studies on influenza virus haemagglutinin. Biochem. Soc. Symp. 55: 155–166.

Gething MJ & Sambrook J (1992) Protein folding in the cell. Nature 355: 33–45.

Grathwohl C & Wuthrich K (1976a) The X-Pro peptide bond as an NMR probe for conformational studies of flexible linear peptides. Biopolymers 15: 2025–2041.

Grathwohl C & Wuthrich K (1976b) NMR studies of the molecular conformations in the linear oligopeptides H-(L-Ala)n-L-Pro-OH. Biopolymers 15: 2043–2057.

Grathwohl C & Wuthrich K (1981) NMR studies of the rates of proline *cis-trans* isomerization in oligopeptides. Biopolymers 20: 2623–2633.

Gunne H, Hellers M & Steiner H (1990) Structure of preproattacin and its processing in insect cells infected with a recombinant baculovirus. Eur. J. Biochem. 187: 699–703.

Haas IG & Wabl M (1983) Immunoglobulin heavy chain binding protein. Nature 306: 387–389.

Haas IG & Meo T (1988) cDNA cloning of the immunoglobulin heavy chain binding protein. Proc. Nat. Acad. Sci. USA 85: 2250–2254.

Hammond C & Helenius A (1994) Folding of VSV G protein: sequential interaction with BiP and calnexin. Science 266: 456–458.

Handschumacher RE, Harding MW, Rice J, Drugge R & Speicher DW (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 226: 544–547.

Haseman CA & Capra JD (1990) High-level production of a functional immunoglobulin heterodimer in a baculovirus expression system. Proc. Nat. Acad. Sci. USA 87: 3942–3946.

Helenius A, Marquardt T & Braakman I (1992) The endoplasmic reticulum as a protein-folding compartment. Trends in Cell Biology 2: 227–231.

Helenius A (1994) How N-linked oligosacharides affect glycoprotein folding in the endoplasmic reticulum. Molecular Biology of the Cell 5: 253–265.

Hochstenbach F, David V, Watkins S & Brenner M (1992) Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-Cell antigen receptors and major histocompatibility complex during their assembly. Proc. Nat. Acad. Sci. USA 89: 4734–4738.

Hsu TA, Eiden JJ & Betenbaugh MJ (1994a) Engineering the assembly pathway of the baculovirus-insect cell expression system. Annals of the New York Academy of Sciences 721: 208–217.

Hsu TA, Eiden JJ, Bourgarel P, Meo T & Betenbaugh MJ (1994b) Effects of co-expressing chaperone BiP on functional antibody production in the baculovirus system. Protein Expression and Purification 5: 595–603.

Hsu TA, Watson S, Eiden JJ & Betenbaugh MJ (1996) Rescue of immunoglobulins from insolubility is facilitated by PDI in the

baculovirus expression system. Protein Expression and Purification 7: 281–288.

Huth JR, Perini F, Lockridge O, Bedows E & Ruddon RW (1993) Protein folding and assembly in vitro parallel intracellular folding and assembly. Catalysis of folding and assembly of the human chorionic gonadotropin alpha beta dimer by protein disulfide isomerase. J. Biol. Chem. 268: 16472–16482.

Jakob U & Buchner J (1994) Assisting spontaneity: the role of hsp90 and small hsps as molecular chaperones. Trends Biochem. Sci. 19: 205–211.

Jarvis DL & Summers MD (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. Mol. Cell Biochem. 9: 214–223.

Jarvis DL, Summers MD, Garcia A & Bohlmeyer DA (1993) Influence of different signal peptides and prosequences on expression and secretion of human tissue plasminogen activator in the baculovirus system. J. Biol. Chem. 268: 16754–16762.

Jeang KT, Giam CZ, Nierenberg M & Khoury G (1987) Abundant synthesis of functional HTLV–1 p40X protein in eucaryotic cells by using baculovirus expression vectors. J. Virology 61: 708–713.

Jenkins N & Curling EMA (1994) Glycosylation of recombinant proteins: problems and prospects. Enzyme Microb. Technol. 16: 354–364.

Kiefhaber T, Quaas R, Hahn U & Schmid FX (1990) Folding of ribonuclease T1. 1. existence of multiple unfolded states created by proline isomerization. Biochemistry 29: 3053–3060.

Kim PS, Bole D & Arvan P (1992) Transient aggregation of nascent thyroglobulin in the endoplasmic reticulum: relationship to the molecular chaperone, BiP. J. Cell Biol. 118: 541–549.

Kim PS & Arvan P (1995) Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding in the endoplasmic reticulum. J. Cell Biol. 128: 29–38.

Knappik A, Krebber C & Pluckthun A (1993) The effect of folding catalysts on the *in vivo* folding process of different antibody fragments expressed in *Escherichia coli.* Bio/Technology 11: 77–83.

Knittler MR & Haas IG (1992) Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. EMBO J. 11: 1573–1581.

Kozutsumi Y, Segal M, Normington K, Gething MJ & Sambrook J (1988) The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature 332: 462–464.

Lamantia ML & Lennarz WJ (1993) The essential function of yeast protein disulfide isomerase does not reside in its isomerase activity. Cell 74: 899–908.

Lang K, Schmid F & Fischer G (1987) Catalysis of protein folding by prolyl isomerase. Nature 329: 268–270.

Lee AS (1987) Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. Trends in Biochem. Sci. 12: 20–23.

Li Z & Srivastava PK (1993) Tumor rejection antigen gp96/grp96 is an ATPase: implications for protein folding and antigen presentation. EMBO J. 12: 3143–3151.

Lilie H, Lang K, Rudolph R & Buchner J (1993) Prolyl isomerases catalyze antibody folding in vitro. Protein Science 2: 1490–1496.

Lilie H, McLaughlin S, Freedman R & Buchner J (1994) Influence of protein disulfide isomerase (PDI) on antibody folding in *vitro,* J. Biol. Chem. 269: 14290–14296.

Lodish HF & Kong N (1991) Cyclosporin A inhibits an initial step in folding of transferrin within the endoplasmic reticulum. J. Biol. Chem. 266: 14835–14838.

Luckow VA (1990) Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: Ho C, Prokop A & Bajpai R (eds) Recombinant DNA Technology and Applications, (pp. 97–149) McGraw-Hill, New York.

Luckow VA (1993) Baculovirus systems for the expression of human gene products. Current Opinion in Biotechnology 4: 564–572.

Lyles MM & Gilber HF(1991) Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: pre-steady-state kinetics and the utilization of the oxidizing equivalents of the isomerase. Biochemistry 30: 619–625.

Marquardt T & Helenius A (1992) Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. J. Cell Biol.117: 505–513.

Mazzarella RA, Srinivasan M, Haugejorden SM & Green M (1990) Erp72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site seqences of protein disulfide isomerase. J. Biol. Chem. 265: 1094–1101.

Melnick J, Aviel S & Argon Y (1992) The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. J. Biol. Chem. 267: 21303–21306.

Melnick J, Dul JL & Argon Y (1994) Sequential interaction of the chaperone BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. Nature 370: 373–375.

Mitchell-Logean & Murhammer (1995) Baculovirus and Gene Expression Conference.

Mitraki A, Haase-Pettingell C & King J (1991) Mechanisms of inclusion body formation. In: Debernardez-Clark E & Georgiou G (eds) Protein Refolding. Vol. 470 (pp. 35–49) American Chemical Society, Washington DC.

Murphy CI, Lennick M, Lehar S, Beltz GA & Young E (1990) Temporal expression of HIV–1 envelope proteins in baculovirus-infected insect cells: implications for glycosylation and CD4 binding. Genetic Analysis, Techniques and Applications 7: 160–171.

Murphy CI, McIntire JR, Davis DR, Hodgdon H, Seals JR & Young E (1993) Enhanced expression, secretion, and large-scale purification of recombinant HIV–1 gp120 in insect cells using the baculovirus egt and p67 signal peptides. Protein Expression and Purification 4: 349–357.

Navarro D, Qadri I, & Pereira L (1991) A mutation in the ectodomain of herpes simplex virus 1 glycoprotein B causes defective processing and retention in the endoplasmic reticulum. Virology 184: 253–264.

Nakhai B, Pal R, Sridhar P, Talwar GP & Hasnain SE (1991) The α subunit of human gonadotropin hormone synthesized in insect cells using a baculovirus vector is biologically active. FEBS J. 283: 104–108.

Noiva R, & Lennarz WJ (1992) Protein disulfide isomerase: A multifunctional protein resident in the lumen of the endoplasmic reticulum. J. Biol. Chem. 267: 3553–3556.

O'Reilly DR, Miller LK & Luckow VA (1992) Baculovirus Expression Vectors. W. H. Freeman and Company, New York.

Ou W, Cameron PH, Thomas DY & Bergeron JJM (1993) Association of folding intermediates of glycoproteins with calnexin during protein maturation. Nature 364: 771–776.

Pasumarthy MK & Murhammer DW (1994) Clonal variation in the Spodoptera frugiperda IPLB-SF21- AE insect cell population. Biotech. Progress 10: 314.

Paul JI, Tavare J, Denton RM & Steiner DF (1990) Baculovirus-directed expression of the human insulin receptor and an insulin-binding ectodomain. J. Biol. Chem. 265: 13074–13083.

Pelham HRB (1989) Control of protein exit from the endoplasmic reticulum. Ann. Rev. Cell Biol. 5: 1–23.

Pigiet VP & Schuster BJ (1986) Thioredoxin-catalyzed refolding of disulfide-containing proteins. Proc. Nat. Acad. Sci. USA 83: 7643–7647.

Pind S, Riordan JR & Williams DB (1994) Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. J. Biol. Chem. 269: 12784–12788.

Potvin B, Kumar R, Howard DR & Stanley P (1990) Transfection of a human α-(l,3)fucosyltransferase gene into Chinese hamster ovary cells, complications arise from activation of endogenous α-(l,3)fucosyltransferases. J. Biol. Chem. 265: 1615–1622.

Puig A & Gilbert HF (1994) Protein disufide isomerase exhibits chaperone and anti-chaperone activity in the oxidative refolding of lysozyme. J. Biol. Chem. 269: 7764–7771.

Rankl NB, Rice JW, Gurganus TM, Barbee JL & Burns DJ (1994) The production of an active protein kinase C-δ in insect cells is greatly enhanced by the use of the basic protein promoter. Protein Expression and Purification 5: 346–356.

Roth RA & Koshland ME (1981) Role of disulfide interchange enzyme in immunoglobulin synthesis. Biochemistry 20: 6594–6599.

Roth RA & Pierce SB (1987) *In vivo* cross-linking of protein disulfide isomerase to immunoglobulins. Biochemistry 26: 4179–4182.

Rothblatt J, Novick P, Stevens T (1994) Guidebook to the Secretory Pathway. Oxford University Press, Oxford.

Rothman JE (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59: 591–601.

Rothman JE (1994) Mechanisms of intracellular protein transport. Nature 372: 55–63.

Schaiff WT, Hruska KA Jr., McCourt DW, Green M & Schwartz BD (1992) HLA-DR associates with specific stress proteins and is retained in the endoplasmic reticulum in invariant chain negative cells. J. Exp. Med. 176: 657–666.

Schmid F (1993) Prolyl isomerase: enzymatic catalysis of slow protein-folding reactions. Annu. Rev. Biophys. Biomol. Struct. 22: 123–143.

Shuler ML, Wood HA, Granados RR & Hammer DA (1995) Baculovirus Expression Systems and Biopesticides. Wiley-Liss Inc., New York.

Siekierka J, Hung SHY, Poe M, Lin CS & Sigal NH (1989) A cytosolic binding protein for theimmunosuppresent FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature 341: 755–757.

Sissom JF & Ellis L (1991) Biosynthesis of the precursor of a soluble human insulin receptor ectodomain in insect sf9 cells infectd with a recombinant baculovirus. Biochem. Biophys. Res. Comm. 177: 764–770.

Smith DF, Larsen RD, Mattox S, Lowe JB & Cummings RD (1990) Transfer and expression of a murine UDP-Gal:β-D-Gal α1,3-galactosyltransferase gene in transfected chinese hamster overy cells. competition reactions between the α1,3-galactosyltransferase and the endogenous α2,3-sialyltransferase. J. Biol. Chem. 265: 6225–6234.

Sridar P & Hasnain SE (1993) Differential secretion and glycosylation of recombinant human chorionic gonadotropin (bhCG) synthesized using different promoters in the baculovirus expression vector system. Gene 131: 261–264.

Stamnes MA, Shieh BH, Chuman L & Zuker CS (1991) The cyclophilin homolog ninaA is a tissue-specific integral membrane protein required for the proper synthesis of a subset of drosophila rhodopsins. Cell 65: 219–227.

Steinmann B, Bruckner P & Superti-furga A (1991) Cyclosporin A slows collagen triple-helix formation *in vivo*: indirect evidence for a physiologic role of peptidyl-prolyl *cis-trans* isomerase. J. Biol. Chem. 266: 1299–1303.

Stern LJ & Wiley DC (1992) The human class II MHC HLA-DR1 assembles as empty αβ heterodimers in the absence of antigenic peptide. Cell 68: 465–477.

Summers MD & Smith GE (1987) Bulletin No. 1555. Texas Agricultural Experiment Station and Texas A&M University.

Tessier DC, Thomas DY, Khouri HE, Laliberte F & Veraet T (1991) Enhanced secretion from insect cells for a foreign protein fused to the honeybee melittin signal peptide. Gene 98: 177–183.

Vogel JP, Misra LM & Rose MD (1990) Loss of BiP/GRP78 functions blocks translocation of secretory proteins in yeast. J. Cell. Biol. 110: 1885–1895.

Vuori K, Pihlajaniemi T, Martilla M & Kivirikko KI (1992a) Characterization of the human prolyl 4-hydroxylase tetramer and its multifunctional protein disulfide-isomerase subunit synthesized in a baculovirus expression system. Proc. Nat. Acad. Sci. USA 89: 7467–7470.

Vuori K, Pihlajaniemi T, Myllyla R & Kivirikko KI (1992b) Site-directed mutagenesis of human protein disulphide isomerase: effect on the assembly, activity, and endoplasmic reticulum retention of human prolyl 4-hydroxlase in Spodoptera frugiperda insect cells. EMBO J. 11: 4213–4217.

Wada I, Ou W, Liu M & Scheele G (1994) Chaperone function of calnexin for the folding intermediate of gp80, the major secretory protein in MDCK cells. J. Biol. Chem. 269: 7464–7472.

Weich H, Buchner J, Zimmermann R & Jakob U (1992) Hsp90 chaperones protein folding in vitro. Nature 358: 169–170.

Weissman JS, & Kim PS (1993) Efficient catalysis of disulphide bond rearrangements by protein disulphide isomerase. Nature 365: 185–188.

Wittrup KD (1995) Disulfide bond formation and eucaryotic secretory productivity. Current Opinion in Biotechnology 6: 203–208.

zu-Putlitz J, Kubasek WL, Duchene M, Marget M, von Specht BU & Domdey H (1990) Antibody production in baculovirus-infected insect cells. Bio/Technology 8: 651–654.

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PART III
ENGINEERING ASPECTS

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Shear sensitivity of insect cells

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Introduction

During the last 15 years, tremendous progress has been made in our ability to produce complex proteins through genetic engineering. One system which has significantly contributed to this advance is the insect-baculovirus process. In addition to the obvious dramatic progress in the understanding of the molecular biology of this system, significant progress has been made in understanding how to culture these cells on a large scale. This progress involves the development of serum-free medium, the optimum conditions for virus infection, the determination of optimum cell lines for productivity, and the deepening understanding of the prevention of damage to the cells from hydrodynamic forces. This chapter will discuss this last topic.

Insect cells have been grown in large-scale cultures (size greater than spinner flask scale) for almost thirty years. The first reported example of large-scale cultures of insects cells in suspension culture is that of Vaughn (1967) using the cell line *Antherae.* In the mid 1970's, fruit flies (*Drosophila melanogaster*) and mosquitoes (*Andes albospictus*) were grown in large scale cultures (Lengyel *et al.*, 1975; Spradling *et al.*, 1975). In 1982, Hink reported the growth of *Trichoplusia ni*, TN368, insect cells in multiliter bioreactors. The largest reported working volume of suspended insect cells was made by Maiorella *et al.* (1988). They reported on the growth of *Spodoptera frugiperda,* SF–9, cells which produce a human macrophage colony-stimulating factor in a 21-liter airlift bioreactor to a cell density of 5×10^6 cells ml^{-1}.

More recently, several reports exist of researchers attempting to increase the cell concentration in bioreactors to improve productivity. Increased yields were obtained through the use of different bioreactor configurations (spin filter or perfusion systems), medium development, or a combination of both (Caron *et al.,* 1994; Binh *et al.*, 1993; Deutschmann & Jaeger, 1994; Kim *et al.*, 1994).

Whether these authors knew it or not, their ability to grow insect cells in suspension culture was due to the fact that some method of protecting the cells from the damaging effects of gas bubbles was present, either through the use of additives or the complete removal of bubbles from the system. As will be discussed below, without protection from bubbles, it is very difficult, if not impossible, to grow cells in aerated cultures.

Hydrodynamics within a bioreactor

In a suspended cell bioreactor, two factors govern the degree of mixing in the vessel: the amount of mixing needed to suspend the cells, and the amount of mixing needed to provide a sufficent, uniform concentration of nutirents to the cells. Historically, impellers within a cylindrical vessel with a flat or half spherical bottom have been used. This type of mixing apparatus is heavily used in the chemical industry and the characteristic operating conditions needed to provide a well-mixed condition on a molecular level are well known.

Besides providing a uniform nutrient concentration for the cells in a bioreactor, sufficient nutrients must be dissolved, either initially or continually, in the suspending medium. This is reasonably easy for most nutrients, even insoluble nutrients such as lipids. However, this is not true for oxygen. There are two main reasons for this problem: the very low solubility of oxygen in water and the relatively high oxygen

consumption rate of intect cell cultures. The solubility of oxygen in growth medium is approximately three orders of magnitude lower than other typical, water soluble nutrients such as glucose. Coupled to this low solubility is a reasonably high oxygen demand. It has been reported that a suspension culture of insect cells can require up to 1.5 mmol l^{-1} h^{-1} of oxygen while mammalian cells usually require oxygen in the range from 0.053 to 0.59 mmol l^{-1} h^{-1} (Karen *et al.*, 1991; Glacken *et al.*, 1983). While this demand is not as high as bacterial cultures, which require up to 90 mmol l^{-1} h^{-1} (Fowler, 1984), this demand, especially in infected insect cells, is sufficiently high to require active methods of oxygen delivery to the cell suspension.

The most common method of oxygen delivery is sparging of air or oxygen bubbles directly into the cell suspension. However, early research in cell culture (Swim & Parker, 1960; Runyan & Geyer, 1963) recognized that the introduction of bubbles into a cell culture system could cause cell damage, and that the addition of specific medium additives, such as Pluronic F–68, could prevent this damage.

Despite these indications of damage as a result of bubble introduction into cell culture, it was generally believed that cells were damaged by the mixing due to impellers (Lee *et al.*, 1988; Backer *et al.*, 1988; Dodge & Hu, 1986). A number of reasons exist for this misconception, not the least of which were studies indicating that anchorage-dependent cells can be removed and killed from microcarriers as a result of mixing in bioreactors.

This misconception was challenged by the work of Oh *et al.* (1989) and Kunas & Papoutsakis (1990a). Both groups presented work which indicated that suspended animal cells (and presumably insect cells) can withstand much higher levels of agitation, generated by impellers, than had been previously believed. However, this observation was true only if cell suspensions were not sparged with gas and care was taken to limit the amount of gas entrainment due to the mixing. The typical rpm for such systems was 50 to 100, but both research groups reported that hybridoma cells could withstand rpm's up to 200. It has also been observed that insect cells can be grown in impeller agitated vessels at rpm's over 100 (Miyake *et al.,* 1977; Cameron *et al.*, 1989; Caron *et al.*, 1990; Agathos *et al.*, 1990).

To further investigate this observation, Kunas & Papoutsakis (1990b) developed a bioreactor in which the top air-medium interface was removed. This removed almost all of the bubbles, since it prevented any bubbles from being entrained as a result of the central vortex which forms around the impeller shaft at high rpm's. With this system Kunas & Papoutsakis were able to generate rpm's of 600 without major cell damage. These results lead them to state "Only when entrained bubbles interact with a freely moving gas-liquid interface, such as exits between the culture medium and gas headspace, does significant cell damage occur."

While the above results were obtained with animal and not insect cells, these results can be applied to insect cell culture. This conclusion is confirmed by the previous observations of Tramper *et al.*, 1986, Mairoella *et al.*, 1988, and Murhammer & Goochee, 1988. Each of these groups reported that without the use of protective additives, it was either impossible or nearly impossible to grow suspended insect cells in bubble columns or airlift bioreactors.

Cell damage associated with bubbles

Two research groups correlated cell damage to bubbles. In a study using SF–9 insect cells, Tramper *et al.* (1988) proposed that associated with each bubble is a "hypothetical killing volume". This volume can be determined using the following equation:

$$k_d = 24FX / (\pi^2 d_b^3 D^2 H) \qquad (1)$$

where

k_d	= first order death rate constant	(s^{-1})
F	= air flow rate into vessel	$(m^3\ s^{-1})$
X	= hypothetical killing volume	(m^3)
d_b	= air bubble diameter	(m)
D	= column diameter	(m)
H	= height of column	(m)

This concept was verified under a variety of conditions: a number of cell lines, vessel aspect ratios, and aeration rates (Martens *et al.*, 1992; Jobses *et al.*, 1991).

Another correlation was developed by Yang & Wang (1992), and Wang *et al.* (1994). This relationship was motivated by the hypothesis that cells are damaged by the breakup and/or coalescence of bubbles in bioreactors which can take place at the sparger, the agitation region, or the air-medium interface at the top of the reactor. In addition, it assumes that cells can

adsorb into an "inactivation zone" around a bubble. The model takes the form of:

$$p = (k_2 s / K)a \qquad (2)$$

where

- p = local specific cell death rate (h^{-1})
- s = equivalent thickness of the inactivation region around a deformed bubble
- a = local specific bubble interfacial area (bubble surface area/medium volume, m^{-1})
- k_2 = intrinsic cell inactivation rate constant (cells m^{-3} h^{-1})
- K = Michaelis-Menton saturation constant for cells adsorbed into an "inactivation zone" around a bubble

As can be observed, the local, specific death rate is linearly proportional to the specific bubble interfacial area. This linear relationship was confirmed when Wang *et al.* (1994) applied this model to the results of Oh *et al.* (1989).

These two correlations provide the following insight into cell damage with respect to bubble rupture: 1) it is linearly proportional to the gas-medium interface area, 2) it is independent of the height and diameter of the vessel, and 3) it is independent of the number of bubbles. This last point implies that cell damage only depends on the size and/or volume of the bubble. As will be discussed below, the size of the bubble plays an important part in cell damage.

Cell damage mechanisms

While the above relationships link cell death to bubbles, they do not indicate the mechanism. In a typical sparged bioreactor, four regions, with respect to bubbles, can be identified: 1) the bubble disengagement region, 2) the bubble rising region, 3) the bubble generation region, and 4) the impeller region if the bioreactor is mixed.

Only one study has been conducted on the bubble generation region (Murhammer & Goochee, 1990). It was observed that if the pressure drop through a sparger in an airlift bioreactor exceeded 3.0 psi, significant cell damage occurred. It was hypothesized that the increased air velocity associated with this higher pressure drop created sufficient hydrodynamic forces to damage the cells. Further work is needed to confirm this hypothesis.

The two correlational studies, mentioned in the previous section, argue against cell damage resulting from simple bubble rising in culture medium. In addition, the photographic work of Orton & Wang (1991) presented experimental results which also indicated that rising bubbles do not damage cells. Using dyes, which stain viable cells one color and dead cells another, they demonstrated in a bubble column that dead cells accumulated around the bubble disengagement region while viable cells remained in the rest of the vessel.

Of the four regions, the bubble disengagement region has been studied the most extensively. Before the research with respect to cells in this region is discussed, a review of the fate of a bubble approaching the disengagement region will be presented.

If few or no bubbles are present at the interface, when a bubble reaches the interface it will penetrate the interface to various degrees, depending on its size, and form a thin film separating the air within the bubble from the gas above the interface. Depending on the compounds in the medium, the bubble film will either rapidly rupture and the bubble will pop, or the bubble film will remain stable and the bubble will stay at the interface for various periods of time. It has been observed under some cell culture conditions that the bubble can remain for more than 30 minutes before rupture (personal observation). If other bubbles are present at the interface, the approaching bubble can become "attached" to the other bubbles forming or contributing to the foam layer at the top of the medium. If a sufficiently large amount of foam is already present, the bubble will slowly penetrate the foam layer from the bottom. Once a bubble is in the foam layer it can slowly coalesce with other bubbles in the foam to form larger bubbles.

The process by which a single bubble at a gas-liquid interface ruptures has been the subject of study for over forty years. In 1968, MacIntyre proposed a schematic diagram, based on photographic evidence, of the bubble burst process (Figure 1). Once the bubble film ruptures, the fluid in this film "rolls up" to form a toroidial ring which rapidly expands to the top of the bubble cavity. Once it reaches the cavity, this liquid, along with a very thin layer surrounding the bubble flows downward toward the bottom of the cavity. Once it reaches the bottom of the cavity, a point of stagnation is reached and two jets are created: one which flows downward into the liquid below the cavity, and one which flows upward into the air above the interface.

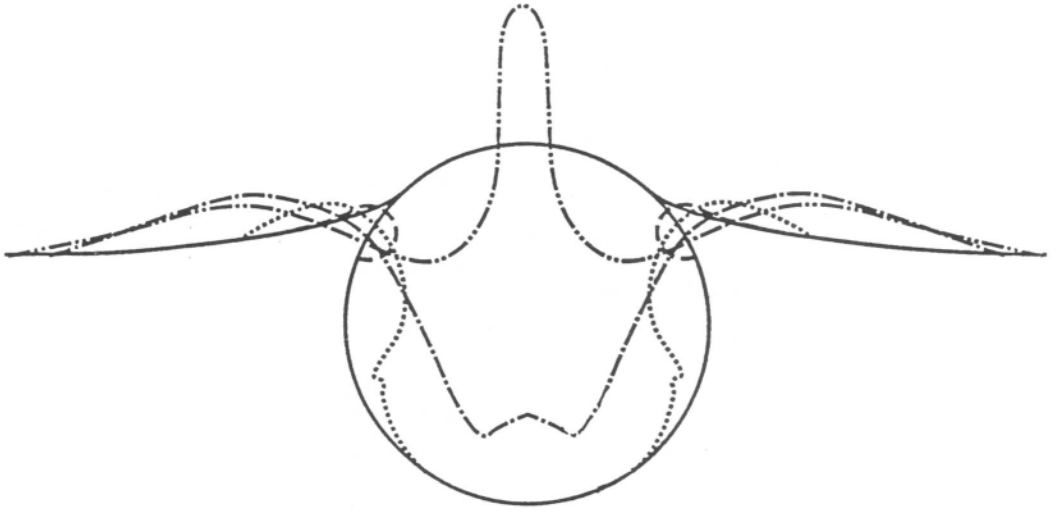

Figure 1. A schematic diagram, based on photographic evidence, of the position of the gas-liquid interface as a bubble, at rest at the top liquid-air interface, ruptures.

MacIntyre, based on experimental studies, reported that the fluid in the upward and lower jets contained liquid from the bubble film and a very thin layer surrounding the bubble cavity. He called this process a boundary-layer "microtome" effect (MacIntyre, 1968; 1972).

Relationship of insect cells to bubbles

While the above discussions summarize the behavior of bubbles in bioreactors, they do not establish the interactions of cells with those bubbles. This relationship was discussed by Bavarian *et al.* (1991) and Chalmers & Bavarian (1991) using high-shutter speed video technology.

Using a specially designed bubble column and microscope/video apparatus, Bavarian *et al.* (1991) reported that insect cells will attach to rising bubbles if no protective additives, such as Pluronic F–68, are present. It was also reported that cells become trapped in the foam layer as they are carried into the foam by the bubbles. Chalmers & Bavarian (1991) continued these observations by reporting that cells remain attached to the bubble film when a bubble comes to rest at a medium-air interface. They further suggested, based on order of magnitude calculations, that the hydrodynamic forces associated with the bubble rupture process are sufficient to damage and/or kill cells.

Continuing this concept, Garcia & Chalmers (1992) collected samples of the upward jet that results when a single bubble, in a cell suspension, ruptures at the gas-medium interface. While greater than 90% of the cells in the cell suspension are viable, only 5 to 10% of the cells in the collected drop were viable. Yet, when Pluronic F–68 was present in the medium, very few of the cells adsorbed to the bubble film and the concentration of cells in the collected sample from the upward jet was much less than that in the cell suspension. These observations confirm that the rupture of bubbles, with cells attached, kills cells.

To quantify this bubble rupture/cell death process, Trinh *et al.* (1994) developed a system in which a large number of consecutive, single bubbles could be created and ruptured. With this system, both the average number of cells in the upward jet as well as the average number of cells killed per rupture could be determined. On average, 1050 cells were killed per 3.5 mm bubble rupture, and the concentration of cells in the upward jet was twice as high as that in the bulk medium, as long as Pluronic F–68 was not present. It was suggested that this 2 ×concentration in the upward jet was the result of the cells preferentially adsorbing to the bubble-medium interface and then being carried away and up into the upward jet by boundary-layer "microtome" effect. This explanation is consistent with the observation that if Pluronic F–68 is present in the medium, cells do not attach to the bubble, and the concentration of cells in the upward jet is much lower

than that in the bulk medium surrounding the bubble. Finally, it was suggested that the "hypothetical killing volume" proposed by Tramper *et al.* (1988) was the thin layer surrounding the bubble cavity in which cells adsorb and through which the "microtome" removes fluid. It is also consistent with Wang & Yang's (1994) absorption hypothesis and model.

The hydrodynamic forces associated with a bubble rupture

In 1972 MacIntyre attempted to analytically solve the equations explaining the bubbble rupture process. At that time he reported that it was not possible, and to this author's knowledge still no analytical solution exists. In addition, computer hardware and software in the 1960's was not sufficiently advanced to provide a numerical solution. However, as a result of the rapid progress in computer technology, two independent numerical solutions have been reported. The first, reported by Boulton-Stone & Blake (1993), used a boundary integral method while the second, reported by Garcia-Briones *et al.* (1994), used a finite-element computer program called Flow 3-D (Flowscience, Los Alamos, NM). Both methods predicted the fluid velocity and shape of the gas-liquid interface as a function of time as the bubble ruptured with the results from both methods compared to experimental observations. Both simulations produced results which were similar to the experimental results. In addition, the technique of Boulton-Stone and Blake predicted the pressure distribution.

To determine the relevance of these simulations to cell damage, both groups also calculated the energy dissipation rate of the rupture process. Energy dissipation rate is a method to compare the complex hydrodynamic forces associated with a bubble rupture to experimental data of cell damage in well-defined flow experiments. Table 1 presents the rates of energy dissipation in which cell damage has been reported in well-defined flow devices, while Table 2 reports the energy dissipation rates calculated by the two simulation techniques. This comparison indicates that the rates of energy dissipation for the rupture of a small bubble (<2.0 mm) calculated by the computer simulations are several orders of magnitude greater than what has been reported to kill cells. It is interesting that several researchers (Handa *et al.*, 1987; Oh *et al.*, 1992) have reported that the rupture of small bubbles is much more damaging to cells than larger bubbles (1 to 2 mm verses 5 mm), an observation which is consistent with the rapid decrease in hydrodynamic forces as the bubble diameter increases.

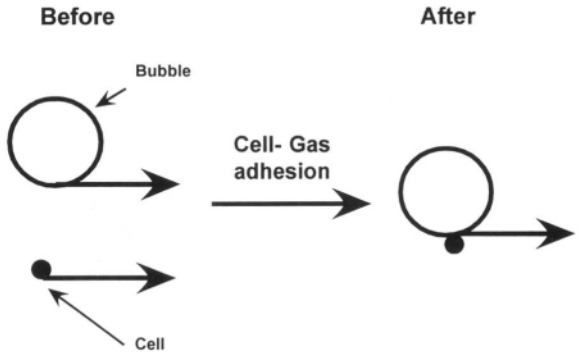

Figure 2. A schematic representation of the process of cell-gas adhesion in a bioreactor.

The mechanism of protection of protective additives

As has been mentioned on several occasions, a number of protective additives have been used to protect cells from bubble damage. Table 3 provides a summary of some of the additives which have been reported to protect cells. Recently Chattopadhyay *et al.* (1995a) evaluated eight of those additives (polyethylene glycol 400, 1000, and 4000; Pluronic F–68; polyvinyl alcohol; Methocel E50 and E4M; and Dextran) with respect to their ability to protect cells from bubble rupture. The central assumption of this work was that nonspecific interfacial phenomena govern the protective capabilities of these additives. This assumption is not surprising since most of the additives used are surface active. It was observed that the additives that reduce the interfacial tension the most (>10 ergs/cm^2) and the fastest (<1 sec) prevented cell adhesion to the bubbles and correspondingly provided the most protection. Two additives met these criteria: Pluronic F–68 and Methocel E50.

The observation that the additives which significantly and rapidly lowered the medium interfacial tension provided the most protection lead Chattopadhyay *et al.* (1995b) to present a thermodynamic relationship to explain this protective effect. The process of bringing a gas bubble, with a characteristic surface tension, γ_{lv}, and a suspended cell, also with a characteristic surface tension, γ_{cl}, together such that a new interface

Table 1. Rates of energy dissipation in which cell damage was reported in well-defined flow devices (From Garcia-Briones *et al.*, 1994)

Cell type	Instrument	Rate of cell damage (% min^{-1})	Rate of dissipation (Jm^{-3} s^{-1})	References
Insect	cone and plate	33.5	3.15×10^4	Goldblum *et al.* (1991)
Hydridoma	concentric cylinder	3.4	2.20×10^4	Schurch *et al.* (1988)
Hydriboma	double cup and bob	*a*	5.81×10^2	Smith (1987)
Mammalian	capillary	16900	4.8×10^7	Augenstein *et al.* (1971)

a At 15 hrs cell viability was 73% (78% at time = 0) as compared with 85% for a control.

Table 2. The rates of energy dissipation for the rupture of different bubble sizes. (from Garcia-Briones *et al.*, 1994; Boulton-Stone *et al.*, 1993)

Bubble diameter (mm)	Total elapsed time (sec)	Maximum energy dissipation rate ($J\ m^{-3}\ s^{-1}$)	
		Garcia-Briones	Boulton-Stone
0.77	5.5×10^{-4}	9.52×10^7	–
1.77	2.0×10^{-3}	1.66×10^7	4.0×10^8
6.32	1.0×10^{-2}	9.40×10^4	8.0×10^3

Table 3. Protective additives used in cell culture with air sparging (from Chattopadhyay *et al.*, 1995)

Protective additives	Cell types	Conc. (wt%)	Methods of cultivation
Pluronic F–68	LS Mouse cells	0.02	Sparged, stirred (Kilburn & Webb, 1968)
	Human Lymphoblastoid	0.05	Aerated, agitated (Mizrahi, 1984)
	NS1 myeloma, Hybridoma, BHK21, Lymphoblastoid	0.1	Bubble column (Handa *et al.*, 1987, 1989)
	Hybridoma (CRL–8018)	0.1	Sparged, agitated (Michaels *et al.*, 1991)
	SF–9	0.1	Airlift (Maiorella *et al.*, 1988)
		0.2	Airlift, sparged, agitated (Murhammer & Goochee, 1988)
Methyl-cellulose	TN–368	0.3	Sparged (Hink & Struass, 1979)
Carboxy-methyl-cellulose	BHK–21	2.4	Sparged, agitated (Telling & Elsworth, 1965)
	Human Lymphoblastoid	0.1	Aerated, agitated (Mizrahi, 1984)
	Dunaliella	0.1	Roux bottle, miniloop reactor (Silva *et al.*, 1987)
HES	Human Lymphoblastoid	0.2	Aerated, agitated (Mizrahi, 1984)
TPB	BHK–21	6.0	Sparged, Agitated (Telling & Elsworth, 1965)
PEG	Hybridoma (CRL–8018)	0.1	Sparged, agitated (Michaels *et al.*, 1991)
PVA	Hybridoma (CRL–8018)	0.2	Sparged, agitated (Michaels *et al.*, 1991)
Bovine Serum Albumin	Hybridoma	1000 mg l^{-1}	Airlift loop reactor (Hulscher & Onken, 1988)
Serum	LS Mouse cells	10	Sparged, stirred (Kilburn & Webb., 1968)
	NS1 myeloma, Hybridoma, BHK21, Lymphoblastoid	10	Bubble Column (Handa *et al.*, 1987, 1989)
	SF–9	10	Airlift (Maiorella *et al.*, 1988)
	Hybridoma (CRL 8018)	10	Sparged, agitated (Kunas & Papoutsakis, 1990)

(Note: HES = Hydroxyethyl Starch, TPB = Tryptose Phosphate Broth, PEG = Polyethylene Glycol, PVA = Polyvinyl Alcohol).

between the cell and gas within the bubble forms, γ_{cv}, is presented in Figure 2. This process can be mathematically represented by the following equation:

$$\Delta F^{adh} = \gamma_{\text{final}} - \gamma_{\text{initial}} = \gamma_{cv} - (\gamma_{lv} + \gamma_{cl}) \quad (3)$$

where ΔF^{adh} is the change in free energy (thermodynamic feasibility) of the process of creating the new interface (adhesion of the cell to the gas-medium interface). If the value of ΔF^{adh} is negative, the process is thermodynamically feasible and spontaneous; if it is positive, it is thermodynamically unfavorable. Chattopadhyay (1995b) next determined the values of γ_{cl} and γ_{cv} from literature and experimental data and a semi-empirical equation of state. They reported that the value of γ_{cl} for 14 different cell lines was always very low, 0.01 to 2.7 erg/cm^2 when compared to the values of γ_{cv} and γ_{lv} which ranged from 56 to 69 erg/cm^2 and 55.5 to 69 erg/cm^2, respectively. By inspection of Equation 3 and the values of γ_{cv}, γ_{lv} and γ_{cl}, it can be observed that when the value of γ_{lv}, the medium interfacial tension, is lower than γ_{cv}, the cell vapor interfacial tension, the adhesion of cells to gas bubbles is thermodynamically unfavorable. This observation confirms the results of Chattopadhyay *et al.* (1995a) which reported that the additives which significantly and rapidly reduced the interfacial tension also prevented cell -bubble adhesion. However, a word of caution at this stage is needed; Chattopadhyay *et al.* (1995b) stated that it is not scientifically sound to attempt to highly quantify cell adhesion using Equation 3. Significant experimental error exists in the determination of γ_{cv} and γ_{cl}, such that only the general trends are significant.

In somewhat similar, but independent studies, Michaels *et al.* (1995a, 1995b) also investigated the interfacial, rheological and protective properties of medium containing additives reported to protect cells from bubble damage. In addition to studying cell-to-bubble-attachment and the relationship of additives to this attachment, they also investigated the effect of additives on the drainage of cells in thin films and the effect of additives on the characteristics and properties of the foam at the medium air interface.

Using an apparatus known as an electronic induction timer, Michaels *et al.* (1995a) determined the relationship between cell-bubble contact time, the number of cells which attached, and the presence of various media additives. While this technique is a kinetic approach, as opposed to the thermodynamic approach of Chattopadhyay *et al.* (1995a,b), Michaels *et al.* (1995a) observed similar results in that Pluronic F–68 and Methocel A15LV had low induction times which indicated that these additives prevented, or greatly decreased, the occurrence of cell adhesion to bubbles as opposed to medium with or without serum. Michaels *et al.* (1995a) also observed that polyvinyl alcohol (PVA) also reduced cell adhesion. Similar results were also observed in the thin film experiments in that cells quickly flowed out of draining films when either Pluronic F–68, Methocel, or PVA were present.

To study the effect that these additives had on foam formation at the air-medium interface, and the presence of cells in that foam, Michaels *et al.* (1995a) conducted foam flotation experiments. A separation factor (ratio of cell concentration in the foam catch to that in the bubble column) was used to determine the effect of various additives. Using this separation factor, Michaels rated various additives with respect to decreasing separation factor (increasing ability to prevent cells from being trapped in the foam layer): polyvinyl pyrrolidone, polyethylene glycol (PEG), serum free medium with no additives, medium with 3% serum, Pluronic F–68, and Methocel A15 LV.

Next, Michaels *et al.* (1995b) investigated the interfacial properties of these additives. As was also observed by Chattopadhyay *et al.* (1995a), the additives which lowered the dynamic surface tension the most (Methocel, F68 and PVA) also reduced cell-to-bubble attachment the most. This reduced dynamic surface tension implies rapid surfactant adsorption, a mobile interface, a lower surface viscosity, and destablization of the foam layer. Still unresolved is the protective mechanism of PEG and PVA since both have been reported to protect cells (Michaels & Papoutsakis, 1991; Michaels *et al.*, 1992), yet both additives cause an increase in cell-to-bubble attachment.

Conclusions

While insect cells can be easily damaged in bioreactors as a result of hydrodynamic forces, it is also relatively easy to prevent this damage. Of several possible damage mechanisms, the best understood and preventable is the attachment of cells to gas-liquid interfaces and the subjection of these attached cells to the hydrodynamic forces and/or physical forces associated with these interfaces. For example, cells attached to gas bubbles in a bioreactor can be transported into the foam layer where they are physically removed from the cell suspension, or they can be killed when the gas bubble

they are attached to ruptures at the medium-air interface at the top of the bioreactor. The easiest method to prevent this damage is through the use of specific surface active compounds, such as Pluronic F–68 or Methocel E–50 which prevent the cells from attaching to the gas-medium interface.

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References

Augenstein DC, Sinskey AJ & Wang DIC (1971) Effect of shear on the death of two strains of mammalian tissue cells. Biotechnol. Bioeng. 13:409–418.

Agathos SN, Jeong Y-H & Venkat K (1990) Growth kinetics of free and immobilized insect cell cultures. Ann. N.Y. Acad. Sci. 589: 372–398.

Binh J, Jarnagin K, Williams S, Chan H & Barnett J (0000) Fed-batch culture of insect cells: a method to increase the yield of recombinant human nerve growth factor (rhNGF) in the baculovirus expression system. J. Biotechnol. 31: 205–217.

Backer M, Metzger L, Slaber P, Nevitt K & Boder G (1988) Large-scale production of monoclonal antibodies in suspension culture. Biotechnol. Bioeng. 32: 993–1000.

Bavarian F, Fan LS & Chalmers JJ (1991) Microscopic visualization of insect cell-bubble interactions. I: Rising bubbles, air-medium interface, and the foam layer. Biotechnol. Prog. 7: 140–150.

Boulton-Stone JM & Blake JR (1993) Gas-bubbles bursting at a free surface. J. Fluid Mech. 154: 437–466.

Cameron IR, Possee RD, Bishop DHL, (1989) Insect cell culture technology in baculovirus expression systems. TIBTECH 7: 66–70.

Caron AW, Archambault J & Massie B (1990) High level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. Biotechnol. Bioeng. 36: 1133–1140.

Caron AW, Tom RL, Kamen AA & Massis B (1994) Baculovirus expression systems scaleup by perfusion of high-density SF–9 cell cultures. Biotechnol. Bioeng, 43: 881–891.

Chalmers JJ & Bavarian F (1991) Microscopic visualization of insect cell-bubble interactions. II: The bubble film and bubble rupture. Biotechnol. Prog. 7: 151–158.

Chattopadhyay D, Rathman JF & Chalmers JJ (1995a) The protective effect of specific medium additves with respect to bubble rupture. Biotechnol. Bioeng. 45: 473–480.

Chattopadhyay, D, Rathman JF & Chalmers JJ (1995b) Thermodynamic Approach to Explain Cell Adhesion to Air-Medium Interfaces. Biotechnol: Bioeng. 48: 649–658.

Deutschmann SM & Jaeger V (1994) Optimization of the growth conditions of Sf21 insect cells for high-density perfusion culture in stirred-tank bioreactor. Enzyme Microb. Technol. 16: 506–512.

Dodge T & Hu W (1986) Growth of hybridoma cells under different agitation conditions, Biotechnol. Letters, 8: 683–686.

Fowler MW (1984) Plant cell culture: natural products and industrial applications. Genet. Eng. Rev. 2: 41–67.

Garcia-Briones MA, Brodkey RS & Chalmers JJ (1994) Computer simulations of the rupture of a gas bubble at a gas-liquid interface and its implications in animal cell damage, Chem. Eng. Sci. 49: 2301–2320.

Garcia-Briones MA & Chalmers JJ (1992) Cell-bubble interactions: Mechanisms of suspended cell damage, Ann. N.Y. Acad. Sci. 665: 219–229.

Glacken MW, Flesichaker RJ & Sinskey AJ (1983) Mammalian cell culture: engineering principles and scale-up, Trends Biotechnol. 1: 102–108.

Goldblum S, Bae Y, Hink WF& Chalmers JJ (1990) Protective effect of methylcellulose and other polymers on insect cells subjected to laminar shear stress. Biotechnol. Prog. 6: 383–390.

Handa-Corrigan A, Emary AN & Spier RE (1989) Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles. Enzyme Microb. Technol. 11:230–235.

Handa A, Emery A & Spier RE (1987) On the evaluation of gas-liquid interfacial effects on hybridoma viability in bubble column bioreactors, Develop. Biol. Standard 66: 241–252.

Hink FH (1982) Production of *Autographa Californica* Nuclear Polyhedrosis virus in cell from large-scale suspension cultures, In: *Microbial and Viral Pesticides,* Kurstak E (ed.) Marcel Dekker, NY.

Hink WF & Strauss E (1979), Suspension culture of the Cabbage Looper (TN–368) cell line, Tissue Cult. Assoc. Man. 5: 1023–1025.

Hulscher M & Onken U (1988), Influence of bovine serum albumin on the growth of hybridoma cells in air-lift loop reactors using serum free medium, Biotechnol. Lett. 10: 689–694.

Jobses I, Martens D & Tramper J (1990) Lethal effects during gas sparging in animal cell culture. Biotechnol. Bioeng. 10: 801–814.

Kamen AA, Tom RL, Caron AW, Chavarie C, Massie B & Archambault J (1991) Culture of insect cells in a helical ribbon impeller bioreactor, Biotechnol. Bioeng. 38: 619–628.

Kilburn DG & Webb F (1968) The cultivation of animal cells at controlled dissolved oxygen partial pressure, Biotechnol. Bioeng. 10: 801–814.

Kim HR, Lee KW, Kim TY, Oh JH, Yang JM, Kang SK & Chung IS (1994) Insect Cell Culture for recombinant β-galactosidase production using a spin-filter bioreactor. J. Microbiol. Biotechnol. 4: 200–203

Kunas KT & Papoutsakis ET (1990a) The protective effect of serum against hydrodynamic damage of hybridoma cells in agitated and surface-aerated bioreactors, J. Biotechnol. 15: 57–70.

Kunas KT & Papoutsakis ET (1990b) Damage mechanism of suspended animal cells in agitated bioreactors with and without bubble entrainment, Biotechnol. Bioeng. 36: 476–483.

Lee G, Huard T, Kaminski M & Palsson B (1988) Effect of mechanical agitation on hydridoma cell growth. Biotechnol. Letters 10: 625–628.

Lengyel J, Spradling A & Penman S (1975) Methods with insect cells in suspension culture II *Drosophila melanogaster.* In: Methods of Cell Biology, Vol. 10, Prescott DM (ed.) Academic Press, NY.

MacIntyre F (1968) Bubbles: a boundary-layer "microtome" for micron thick samples of a liquid surface, J. Phys. Chem. 72: 589–592.

MacIntyre F (1972) Flow patters in breaking bubbles, J. Geophys. Res. 77:5211–5228.

Martens DE, de Gooijer CD, Beuvery EC & Tramper J (1992) Effect of serum on hybridoma viable cell density and production of monoclonal antibodies in CSTR's and on shear sensitivity in air-lift loop reactors. Biotechnol. Bioeng. 39: 891–897.

Maiorella B, Inlow D, Shauger A & Harano D (1988) Large scale insect cell-culture for recombinant protein production. Bio/technol. 6, 1406–1410.

Michaels JD, & Papoutsakis EF (1991) Polyvinyl alcohol and polyethylene glycol as protectants against fluid-mechanical injury of freely-suspended animal cells. J. Biotechnol. 19: 241–258.

Michaels JD, Kunas, KT & Papoutsakis ET (1992) Fluid Mechanical damage of freely suspended animal cells in agitated bioreactors: Effects of dextran, dreivatized celluloses, and polyvinyl alcohol. Chem. Eng. Comm. 118: 341–360.

Michaels JD, Nowak JE, Mallik AK, Koczo K, Wasan DT & Papoutsakis ET (1995a) Analysis of Cell-to-Bubble Attachment in Sparged Bioreactors in the Presence of Cell-Protecting Additives. Biotechnol. Bioeng. 47: 407–419.

Michaels JD, Nowak JE, Mallik AK, Koczo K, Wasan DT & Papoutsakis ET (1995b) Interfacial Properties of Cell Culture Media with Cell-Protecting Additives. Biotechnol. Bioeng. 47: 420–430.

Miyake T, Saigo K, Marunouchi T & Shiba T (1977) Suspension culture of Drosophila cells employing a gyratory shaker, In Vitro, 13: 245–251.

Mizrahi A (1984), Oxygen in human lymphoblastoid cell line cultures and effect of polymers in agitated and aerated cultures, Devel Biol Std 55: 93–102.

Murhammer DW & Goochee CF (1988) Scaleup of insect cell cutlures: protective effects of Pluronic F–68. Bio/technol. 6: 1411–1415.

Murhammer DW & Goochee CF (1990) Sparged animal cell bioreactors: mechanisms of cell damage and Pluronic F–68 protection. Biotechnol. Prog. 6: 391–397.

Oh SKW, Nienow AW, Al-Rubeai & Emery AN (1989) The effect of agitation intensity with and without continuous sparging on the growth and antibody production of hybridoma cells. J. Biotechnol. 12: 45–62.

Oh SKW, Nienow AW, Al-Rubeai M & Emery AN (1992) Further studies on the culture of mouse hybridomas in an agitated bioreactor with and without continuous sparging. J. Biotechnol. 22: 245–270.

Orton D & Wang DIC (1991) Fluorescent visualization of cell death in bubble areated bioreactors, Cell Culture Engineering III, Engineering Foundation, Feb. 2–7.

Runyan W & Gyer R (1963) Growth of L cell suspensions on a Warburg apparatus. Proc. Soc. Biol. Med. 112: 1027–1030.

Schurch U, Kramer H, Einsle A, Widmer F & Eppenberger HM (1988) Experimental evaluation of laminar shear stress on the behaviour of hybridoma mass cell cultures producing monoclonal antibodies against mitochondrial creatine kinase. J. Biotechnol. 7: 179–184.

Silva HJ, Cortinas T & Ertola RJ (1987) Effect of hydrodynamic stress on *Dunaliella* growth. J. Chem. Technol. Biotechnol. 40: 41–49.

Smith CG, Greenfield PF & Randerson DH (1987) A technique for determining the shear sensitivity of mammalian cells in suspension culture. Biotechnol. Tech. 1: 39–44.

Spradling A, Stinger RH, Lengyel J & Penman S (1975) Methods with insect cells in suspension culture. I. *Aedes albopictus.* In: Methods of Cell Biology, Vol. 10, Prescott DM (ed.) Academic Press, NY.

Swim H & Parker R (1960) Effect of Pluronic F–68 on growth of fibroblasts in suspension on rotary shakers. Proc. Soc. Biol. Med. 103: 252–254.

Telling RC & Elsworth R (1965) Submerged culture of hamster kidney cells in a stainless steel vessel, Biotechnol. Bioeng. 7: 417–434.

Tramper J, Williams J & Joustra D (1986), Shear sensitivity of insect cells in suspension, Enzyme Microb. Technol. 8: 33–36.

Trinh K, Garcia-Briones MA, Hink FH & Chalmers JJ (1994), Quantification of damage to suspended insect cells as a result of bubble rupture, Biotechnol. Bioeng. 43: 37–45.

Vaughn JL (1967) Growth of insect cell lines in suspension culture, In: Proc. Int. Colloq. Invertebr. Tissue Cult. 2nd Tremezzo Como, Italy. Wang NS, Yang JD, Calabrese RV, Chang KC (1994) Unified modeling framework of cell death due to bubbles in agitated and sparged biorectors. J. Biotechnol. 33: 107–122.

Yang JD & Wang NS (1992) Cell Inactivation in the Presence of Sparging and Mechanical Agitation, Biotechnol. Bioeng. 40: 806–816.

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Insect cell bioreactors

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Key words: insect cells, bioreactors, parameters, production

Introduction

In its present state of development, the insect cell-baculovirus expression system is mostly exploited for the quick and abundant expression of heterologous proteins that are intended for testing of biological activities, for structure-function studies, for use as diagnostics or for the elaboration of candidate vaccines for humans and animals. However, with accumulating insights regarding the faithfulness of post-translational processing and with a better understanding of the many factors affecting protein productivity, it is expected that the insect cell-baculovirus system will emerge as a competitive tool for the commercial production of preventive and therapeutic biopharmaceuticals on an equal footing with microbial or mammalian expression systems currently used in industry. As the usefulness of this technology has become more evident in the last few years, there is an increasing need to develop large-scale systems of insect cell cultivation in order to ensure both volume and consistency of production.

The success of commercially viable production schemes using insect cell cultures depends on several issues, including the development and characterization of superior insect cell lines adapted for growth in large-scale vessels, the formulation of inexpensive and convenient insect cell culture media, especially chemically defined serum-free and protein-free media, and the design of appropriate large-scale bioreactors together with the optimization of the conditions favoring high productivity in these systems (Agathos, 1993). This article addresses some of the most characteristic recent developments in the latter area of insect cell bioreactors.

Large-scale insect cell propagation

Issues of an engineering nature in the design of insect cell culture processes were first confronted almost two decades ago in conjunction with the commercial production of wildtype baculoviruses for use as biopesticides (Hink & Strauss, 1976; Vaughn, 1976). The adaptation of insect cell lines to propagation in suspension culture using scalable reactors was first undertaken at that time. During these early studies, the importance of parameters such as pH, oxygen tension, agitation mode and intensity, foaming, and cell aggregation was recognized, and mostly empirical attempts were made to optimize the cultivation towards obtaining the highest virus titer. For instance, the cultivation of *Trichoplusia ni* (cell line TN-368) in 100-ml aerated spinner flasks was improved in terms of final cell density by direct air sparging, pH adjustment and addition of 0.01% methylcellulose to the medium to prevent cell clumping (Hink & Strauss, 1976). Further experimentation with larger reactors of stirred tank design was done with incremental technical interventions: for successful propagation of TN-368 cells in 2–3 liters of liquid working volume, the microbial-type stirred jar reactor had to be equipped with marine impellers, 0.02% silicone antifoam had to be added and the level of dissolved oxygen (DO) had to be maintained between 15 and 50% by judiciously controlling the flow rates of the sparged air input so that foaming and damage to cells could be minimized (Hink & Strauss, 1980; Hink, 1982).

More systematic attempts to identify the most critical factors likely to affect large-scale propagation of cultured insect cells started appearing in the mid- and late 1980's (Tramper *et al.*, 1986; Maiorella *et al.*,

1988; Wu *et al*., 1989; Agathos *et al*., 1990; Caron *et al.*, 1990; Shuler *et al*., 1990). A critical reading of such reports shows that the factors in need of optimization with direct implications for bioreactor design and operation are multiple and include: cell density, cell viability, growth stage, multiplicity of infection (MOI), time post infection for harvesting, cell nutrition (media ingredients, serum or substitutes) and timing of feeding, dissolved oxygen concentration (DO), temperature, pH and osmotic pressure of media. A concrete definition and understanding of the qualitative and quantitative influences of these parameters, one by one, on virus or protein productivity by the insect cell – BEV system can lead to optimized cultivation and infection protocols that eventually could be more widely applicable and less system- (e.g. cell line-) specific. Some specific examples of studies on selected parameters (oxygen, MOI, temperature, pH and osmolality) in the context of insect cell bioreactor cultivation are given in the next section.

For insect cell cultivation two central sets of considerations underlie bioreactor design and scale-up. First, the need to satisfy the aerobic respiration requirements of the proliferating and infected cell population under the physicochemical, mechanical and geometric constraints imposed on the devices used for homogeneous mixing and oxygen supply to the culture, while at the same time safeguarding the cells' integrity from hydrodynamic shear forces. Second, the quest for maximal volumetric productivity in virus or protein product (amount per unit volume of bioreactor), which may be achieved through increased specific gene expression capacity (or viral particle formation rate) per cell and/or increased useful cell density per unit reactor volume over the course of the process.

The satisfaction of the insect cells' oxygen demand upon bioreactor culture and scaleup requires an understanding of the hydrodynamic forces exerted upon the cells in the cource of agitation and sparging. Extensive work on this topic is summarized in excellent recent reviews (Papoutsakis, 1991; Tramper *et al*., 1993; Chalmers, 1994) and will not be covered here except to signal some concrete consequences for bioreactor configuration: (a) media additives such as Pluronic F-68 and other polymers or serum ingredients are effective in preserving high viability in agitated and bubble-aerated vessels, primarily by physically preventing the adhesion of cells to the surfaces of bubbles and thus avoiding cell death by the tremendous local forces on rupturing bubbles in gas-liquid interfaces, (b) establishing a judicious regime of air bubble diameters to diminish bubble coalescence is beneficial for cell viability in directly sparged stirred or airlift bioreactor systems, (c) physical separation of cells and air bubbles or use of bubble-free aeration in reactor designs (split-flow airlift, membrane-oxygenated stirred tank, etc) protects cells from air bubble-induced damage and (d) a 'killing volume' or equivalent volume of liquid surrounding a bubble before bursting is directly proportional to the first-order death constant of the cells, can be calculated from geometric and operational parameters for a given cultivation system in order to design optimally bubble columns or airlift reactors, and may lead to the selection of the aspect (height to diameter) ratio as a key to scale-up.

In order to maximize productivity, high cell density cultures should be an obvious answer to bioreactor design, in conjunction with the appropriate choice of operation pattern, i.e., batch, repeated (fed-)batch or continuous (e.g. perfusion) process. However, higher cell densities do not necessarily lead to correspondingly higher virus or recombinant protein production. Insect cells may exhibit a type of contact inhibition with respect to susceptibility to baculovirus infection and, consequently, affect adversely viral and protein yield (Wood *et al*., 1982; Wickham and Nemerow, 1993). A general observation in cultured insect cells is that there is an optimal range of cell density for infection, and that infection at cell densities above this critical range leads to a decline in productivity per cell (Caron *et al*., 1990; Lindsay and Betenbaugh, 1992; Wickham *et al*., 1992; Hensler and Agathos, 1994). This phenomenon is currently thought to be due possibly to depletion of medium nutrients (glucose, glutamine, other amino acids and growth factors) or of oxygen or to the accumulation of inhibitory metabolic byproducts (lactate, ammonia, or uric acid) or to the relative prevalence of cells in the various phases of the cell cycle, rather than to contact inhibition (Caron *et al*., 1990; Lindsay and Betenbaugh, 1992; Zhang *et al*., 1993, 1994b; Kioukia *et al*., 1995).

Confirmation of the underlying causes for this conflict between cell density and intrinsic productivity has led recently to nutritional control (feeding) strategies in bioreactor-based insect cell process, that are briefly reviewed at a later section of this article.

Parameters affecting insect cell bioreactor operation

Given our still incomplete knowledge of insect cell behavior in culture, it is important to identify a rela-

tively limited number of rational criteria for the development of efficient recombinant protein production schemes using a BEV-insect cell system that is scalable to a production facility. A scalable system has to be inherently simple and reliable in operation and control, for easy validation in a production facility. The system must also be reproducible and prolific in terms of hererologous protein production. At the present time suspension cultures appear to be the most suitable configuration in terms of reliability and scalability according to reasonably well-established engineering principles. However, alternative, often empirical designs, are also proposed for specific insect cell processes. This is because frequently highly productive cell lines may be extremely attractive on a small scale for the expression of a given gene product in monolayer cultures (e.g., in T-flasks or on microcarriers) but are generally not easily propagated in suspension in large-scale agitated and sparged bioreactors without special effort (see below). Because of the interdependence among the set of conditions affecting the intrinsic protein expression level of a given insect cell line and those conditions influencing the adaptability of the cell line to suspension culture and to serum-free media, a compromise strategy can help reach the global target of maximal protein productivity and titer.

It stands to reason, then, that a comparative assessment of protein productivity among different insect cell lines must be done not only in laboratory systems but in scalable bioreactors (Agathos, 1993, 1994). For example, when *T. ni* (line TN 368) and *S. frugiperda* (line Sf9) were compared with regard to their productivities in spinner flasks using the same medium, it was found that the *T. ni* cells produced 30% more ß-galactosidase on a per cell basis and twice as much on a volumetric basis than the *S. frugiperda* cells (Ogonah *et al.*, 1991). On the other hand, *T. ni* cells are far more susceptible to damage by hydrodynamic shear than *S. frugiperda* cells (Goldblum *et al.*, 1990; Ogonah *et al.*, 1991) and, therefore, unless fully adapted to withstand the rigors of bioreactor cultivation, the former type of cells may not be able to express their full potential on an industrial scale. A case in point is the anchorage-dependent *T. ni* strain BTI Tn-5Bl-4 (commonly referred to as Tn5 or "High Five"), well-known for its superior productivity of glycosylated and secreted proteins, which tends to grow poorly in suspension (Wickham & Nemerow, 1993). Fortunately, even such prolific cells as Tn5 can now be adapted to suspension cell culture (Schlaeger *et al.*, 1994; Depinto & Familetti, 1994) and, therefore, can be propagated and scaled up in standard design bioreactors. This system proved between 5- and 15-fold more prolific than either Sf9 or Sf21 cells of *S. frugiperda* in expressing such proteins (e.g., a mutant IgE receptor, a soluble IgE fragment, a soluble Ile-2 receptor and the p40 subunit of mouse Ile-12 receptor) (Depinto & Familetti, 1994). The adaptation of the Tn5 cell line to suspension growth can be long and arduous (e.g., over 8 months, Schlaeger *et al.*, 1994), encompassing initial growth in large cell aggregates and slow, progressive switching to single-cell growth. Preliminary data on expression of recombinant receptor-type proteins in this cell line after adaptation to growth in suspension were encouraging when scaled up in 60-liter airlift fermentors using an inexpensive serum-free medium formulation (Schlaeger *et al.*, 1994). This specially formulated growth medium was made by mixing 90% of the newly concocted, low-cost SF-1 medium (Schlaeger *et al.*, 1993) and 10% of the commercially available medium ExCell 401 (JRH Biosciences, Lenexa, KS).

Below are given some examples of selected parameters whose study and optimization in bioreactors has been undertaken in the last few years with a potential favorable impact on product maximization.

Oxygen

Although oxygen consumption in animal cells varies over a wide range with reported OUR values from 1.4×10^{-17} moles.cell^{-1}.s^{-1} to 12.5×10^{-17} moles.cell^{-1}.s^{-1} (Fleischaker & Sinskey, 1981), most mammalian cells in culture are situated towards the lower end of this range and most insect cells tend to occupy the mid- and higher values: for example, hybridomas are reported to exhibit $1.55–3.4 \times 10^{-17}$ mol.cell^{-1}.s^{-1} and insect cells $3.5–9.9 \times 10^{-17}$ mol.cell^{-1}-s^{-1} (Kioukia *et al.*, 1995). A number of OUR values for insect cells in culture have been reported under widely differing conditions. For instance, cell line Tn-368 exhibited an OUR of 6.2×10^{-17} mol.cell^{-1}.s^{-1} (Streett & Hink, 1978), while Sf9 cells were reported variously to require 4.3×10^{-17} mol.cell^{-1}.s^{-1} (Maiorella *et al.*, 1988), 5.6×10^{-17} mol.cell^{-1}.s^{-1} (Kamen *et al.*, 1991), 1.2×10^{-16} mol.cell^{-1}.s^{-1} (Reuveny *et al.*, 1993b) or 9.7×10^{-17} mol.cell^{-1}.s^{-1} (Hensler & Agathos, 1994). The variations might partially reflect the diverse growth media used by the different groups. In a comparative study of growth and metabolism of Sf9 cells in serum-supplemented medium (Hink's TNM-FH with 5% fetal bovine serum (FBS)) and serum-free medium (ExCell

401) in 250-ml stirred reactors, we have found that the specific OUR for exponential growth in the former medium was 6.2×10^{-17} mol.cell^{-1}.s^{-1} and 9.7×10^{-17} mol.cell^{-1}.s^{-1} in the latter (Hensler *et al.*, 1994). This difference represents a 50% increase in intrinsic respiration rate for the same insect cell line growing in the absence of serum. An even bigger difference between specific OUR of Sf9 cells propagated in serum-containing medium (basal IPL-41 with glucose and 10% fetal calf serum) and in serum-free medium (ICSF-WB, BioWhittaker) was noted by others (Reuveny *et al.*, 1992): 6.1×10^{-17} moll.cell^{-1}.s^{-1} and 1.2×10^{-16} mol.cell^{-1}.s^{-1}. Such marked differences may reflect selection of a clone with altered respiration capacity in the cource of adaptation to serum-free growth or a direct metabolic effect of the media components on cell respiration, which is intensified during increased rates of biosynthesis in the absence of growth factors normally found in serum.

As a general trend, oxygen consumption by cultured insect cells has been observed to increase upon infection with baculovirus (Streett & Hink, 1978; Kamen *et al.*, 1991: Reuveny *et al.*, 1993b; Hensler & Agathos, 1994), even though there is at least one report of a decrease in OUR after infection (King *et al.*, 1992). A number of reported basal values together with the percentage of OUR change upon infection is given in Table 1. The increase is transient and the rate of oxygen consumption falls progressively as the cytopathic effect of the virus on the cells takes hold and the cells die (Hensler & Agathos, 1994). Thus, the reported variation (from less than 10% to over 100%) in the level of OUR change upon infection may reflect a number of unaccounted influences, including the cell density and physiological state (e.g., relative distribution of cells in the cell cycle), the MOI used and also the time of OUR measurement (Kioukia *et al.*, 1995). It appears that enhanced oxygen demand is a consequence of viral infection and replication rather than a result of gene expression (Schopf *et al.*, 1990). However, the correlation of the OUR profile with the progress of infection may be of interest as a window for closer monitoring of the infection process (see below).

When left uncontrolled, DO is expected to vary in response to the prevailing OUR, assuming that the oxygen supply in the bioreactor remains substantially stable. The influence of the DO in itself is still unclear, despite a number of reports in the literature pointing out its potential significance for recombinant protein productivity (Lindsay and Betenbaugh, 1992; Scott *et al.*, 1992). This can only be studied effectively in bioreactors with controlled DO levels. Such indications are summarized in Table 2. DO control in a bioreactor cultivation of insect cells should ensure that growth and product formation during infection are not limited by oxygen availability and that oxygen does not harm the cells via free radicals generated at excessive concentrations. DO in a roller bottle culture of Sf9 cells infected with a wild type baculovirus was found to be 25% lower than in an uninfected control (Weiss *et al.*, 1982). According to Klöppinger *et al.* (1990, 1991), the highest cell density in a bioreactor was obtained at a DO controlled at 40% of air saturation, while the yield in viral polyhedra was considerably lower when the DO was kept at 20% compared to values of 40% and higher. The DO had to be held at 70% for optimal performance by the less commonly used clone Sf21 of *Spodoptera frugiperda* (Deutschmann & Jäger, 1994). Our own work indicates that, for Sf9 cells in ExCell 401 serum-free medium, there is no substantial influence of DO either on growth or on ß-galactosidase final titer for a wide range of DO values (5-100%) in 250-ml stirred reactors with stringent DO control (Hensler *et al.*, 1994).

To the extent that a limiting DO concentration is implicated in the elicitation of protease activity in insect cell cultures, as preliminary results with Sf9 cells seem to suggest (Wang *et al.*, 1995), controlling DO may contribute to better recombinant protein production through protection from proteolytic attack. Such a protection from protein degradation may also result from pulse additions of protein hydrolyzates early in the post-infection period (Kirn & Familetti, 1994).

Multiplicity of infection

The multiplicity of infection (MOI) has been recognized as potentially affecting the production outcome in insect cell – baculovirus systems, but still no firm conclusions emerge from the literature, despite considerable experimental work and structured modelling of the infection process (de Gooijer *et al.*, 1989; Licari & Bailey 1991, 1992; Zhang *et al.*, 1994b; Kioukia *et al.*, 1995). Some workers have noted that protein production is relatively insensitive to MOI level in the range from 1 to 20 (Maiorella *et al.*, 1988; Murhammer & Goochee, 1988; Neutra *et al.*, 1992). Others report that a strong positive correlation (semilogarithmic relationship) exists between heterologous protein yield and MOI in T-flask cultures infected in mid- to late exponential growth phase but that for cultures infected in early exponential growth phase, the MOI

Table 1. Comparative data on specific Oxygen Uptake Rate (OUR) during insect cell infection (adapted from Kioukia *et al.*, 1995)

OUR after infection (10^{-17} mol.cell^{-1}.s^{-1}	Days post-infection	% change of OUR before/after infection	Cell type	Virus Type	References
20	1	+100	TN 368	WT-AcNPV	Streett & Hink, 1978
7.0	1	+14	Sf9	WT-AcNPV	Schopf *et al.*, 1990
5.28	1	+55	Sf9	WT-AcNPV	Kioukia *et al.*, 1995
4.6	1	+7	Sf9	M-CSF	Maiorella *et al.*, 1988
8.5	1	+47	Sf9	ß-gal	Schopf *et al.*, 1990
3.2	1	+30	Sf9	ß-gal	Kioukia *et al.*, 1995
3.1	3	–56	Sf9	ß-gal	King *et al.*, 1992
9.7	1	+50	Sf9	ß-gal	Hensler & Agathos, 1994
12	1	+100	Sf9	ß-gal	Reuveny *et al.*, 1993b
13.8	1	+40	Sf9	Bac BRV6L	Kamen *et al.*, 1991
11.1	1	+27	Sf9	BHC11	Scott *et al.*, 1992

Table 2. Effect of controlled Dissolved Oxygen (DO) level on insect cell growth and product formation in bioreactors

Cell Line	DO Level (% air sat.)	Growth	Protein Titer	Viral Titer	References
TN-368	100	+++			Hink (1982)
	50	+++			
Sf9	110	+	+		Jain *et al.* (1991)
	65	+++	+++		
	10	+	+		
Sf9	80	+		+++	Klöppinger *et al.*,
	60	+		+++	(1990, 1991)
	40	+++		+++	
	20	++		+	
Sf9	100	+++	+++		Hensler *et al.* (1994)
	50	+++	+++		
	10	+++	+++		
	5	+++	+++		
Sf21	100	+	+		Deutschmann &
	70	+++	+++		Jäger (1994)
	40	+	+		
Bm5	60	++	NR [1]		Zhang *et al.* (1994a)
	40	++	+++		
	30	+++	+++		
	20	++	NR		
	10	+	NR		

[1] NR: Not reported.

does not affect protein production (Licari & Bailey, 1991).

In suspension cultures of Bm5 cells from *Bombyx mori* infected in mid-exponential phase (2.67×10^6 cell.ml^{-1}) expression rates and final titers of a reporter gene product, recombinant chloramphenicol acetyltransferase (CAT), were virtually the same with MOI ranging from 5 to 20 (Zhang *et al.*, 1994b). In this

work, the final CAT yield at an MOI of 1 was lower than at any MOI greater than 1 and specific productivities for MOI of 0.1 and 1 were equal to each other but lower than those obtained with MOIs of 5, 10 and 20 (Zhang *et al*., 1994b). According to Bédart & coworkers (1994b) for Sf9 cells infected in early exponential phase (at densities up to 3×10^6 cell.ml^{-1}) MOI had no marked influence on specific ß-galactosidase yield, although there was a weak tendency for greater protein volumetric titers of lower MOIs. The authors invoke the explanation proposed by Licari & Bailey (1992), namely that greater titers at lower MOIs are the consequence of greater cell densities that occur because of the continued division of uninfected cells following virus addition, whereas in cultures infected at high MOIs such multiplication is restricted, resulting in lower cell densities. Infections at higher cell densities (5.5 and 7.07×10^6 cells.ml^{-1}) showed a positive correlation between specific protein yields and MOIs, provided that medium was replaced (Bédart *et al*., 1994b). Finally, in another recent study of the possible influence of MOI upon viral infectivity and protein yield in Sf9 cells grown in suspension, it was found that if cells were infected in post-exponential (stationary) phase in fresh medium, an MOI of 50 compared to an MOI of 1 enhanced the rate of infection (rate of polyhedra development) but not the final infectivity (final % of infected cells), while cells from exponential phase infected with recombinant virus showed that maximum ß-galactosidase per ml and per cell as well as viral tiler were not significantly different between these two MOIs (Kioukia *et al*., 1995). In the same study, maximal ß-galactosidase production in cells infected at an MOI of 10 was 35% higher for exponential-phase cells than stationary-phase cells. These authors point out that differences in infectivity of cells at different stages in batch growth may well reflect the relative percentage of S-phase cells (most susceptible to infection) in each of these stages (Kioukia *et al*., 1995).

In sum, the MOI can be an important parameter for optimization of bioreactor-based protein or virus production by the insect cell-baculovirus system, depending on the nutritional and growth cycle stage of the cells at infection.

Other physical factors

Temperature may also be used as a control parameter to steer bioreactor cultivation of insect cells for optimal productivity. In temperature shift-down experiments from 33 °C to 27 °C, infected Sf9 cells were allowed to switch on viral growth of a temperature-sensitive baculovirus in one serum-free and two serum-supplemented media (King *et al*., 1991). The lowest virus and CAT titers were obtained in the serum-free medium, while in all three media the lowest MOI (0.01) gave the greatest virus and CAT titers, in contrast to the trend found in constant-temperature cultivation and infection at 27 °C (the highest MOI giving the highest titers) (King *et al*., 1991). Under the latter conditions both serum-free and serum-containing media performed equally well, hence, the authors concluded that serum may act as a thermal buffer against virus inactivation in high-temperature culture.

Two different recombinant proteins, ß-galactosidase and cerebrosidase were produced equally well in shake-flask culture of Sf9 cells at 22, 25 and 27 °C, while production at 30 °C was significantly lower (Reuveny *et al*., 1993b). In this work it was shown that this decrease is due to oxygen limitation and, therefore, a production strategy may involve culturing the cells at their optimal temperature for growth (e.g., 27 °C) and then lowering the temperature during the infection phase to alleviate the oxygen limitations possible due to increased OUR during the early part of the infection phase.

The optimal pH for the growth of most insect cells in culture lies between 6.2 and 6.3, i.e. it is rather acidic compared to the growth pH required by cultured mammalian cells. Left uncontrolled, the pH of Sf9 cell cultures has been reported to go through a minimum of 6.05 in serum-containing medium (TNM-FH with 5% FBS) and 5.90 in serum-free medium (ExCell 401) in stirred-tank bioreactors (Hensler *et al*., 1994). A similar value for a minimum pH was noted in Sf9 cultivation in an airlift reactor with SF900 (Gibco/Life Technologies, Grand Island, NY) serum-free medium (King *et al*., 1992). The upward concave trend of the pH profile is indicative of lactate production early in the culture, which is progressively consumed, while ammonia is accumulating later in the batch. For Bm5 cell culture, it was found that the optimum pH for growth was 6.10–6.30 and that a pH of 6.40 resulted in less than half the recombinant CAT protein yield per 10^6 cells compared to culture at a pH of 6.30 (Zhang *et al*., 1994a). These and other indications in the literature suggest that pH in growth and production phases must be optimized and possibly be kept under light control in bioreactors, especially in high-density large scale cultivations.

Finally, osmolality of insect cell media is one more factor in need of optimization. Insect cells show a

relatively high tolerance to asmotic pressure while most media range in osmolality between 320 and 375 $mosm.kg^{-1}$ (Agathos *et al.*, 1990). A recent study of osmolality effects on Bm5 cells in bioreactor culture under well-controlled conditions showed that the maximum specific growth rate, μ, was achieved at a medium osmolality of 370 $mosm.kg^{-1}$ while more than 90% of μ was reached with osmolalities between 350 and 385 $mosm.kg^{-1}$ (Zhang *et al.*, 1994a).

Insect cell bioreactors for production scale applications

Some examples of recently reported large-scale reactor cultivations are given in Table 3 as an indication of current trends in insect cell bioreactor design and scale-up. A brief discussion of some systems that have been proposed and tested with a view to eventual production-scale application is given below. Bioreactors can be distinguished on the basis of cell culturing method (suspension, attachment to a solid surface, immobilization by entrapment or encapsulation) and on the basis of the mode of bioprocessing (batch, repeated batch, continuous).

Attached cell culture systems

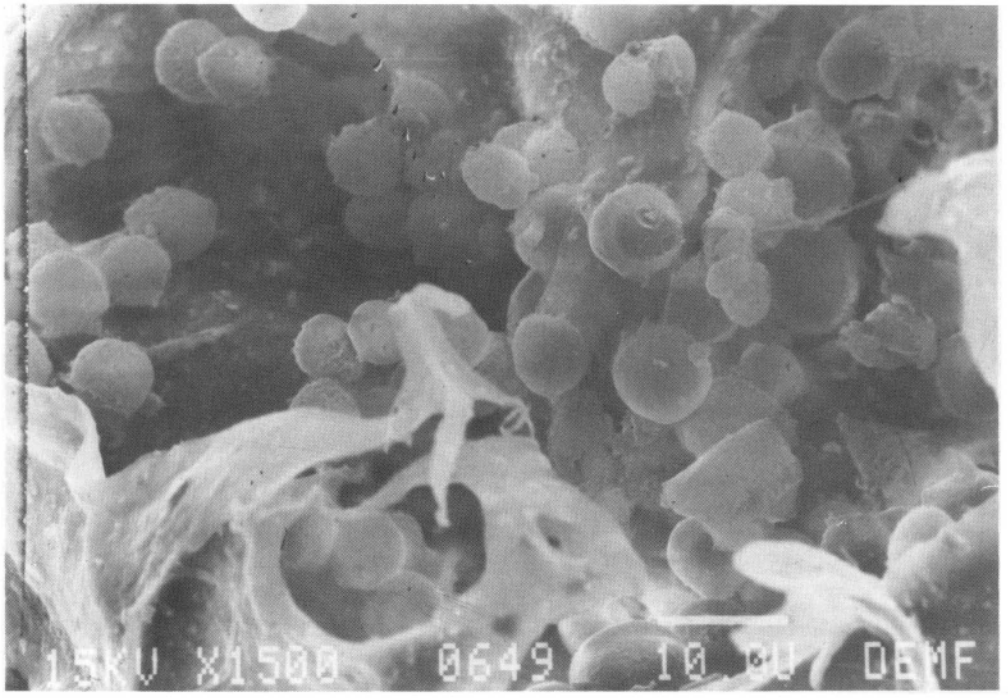
As a rule, most types of insect cells tend to attach loosely to solid surfaces for growth. The cells can be detached by gentle agitation or with a silicon rubber scraper or similar device ("rubber policeman"), but can also be removed by enzymatic treatment, in a manner analogous to trypsinization of anchorage-dependent mammalian cells. For example, Weiss & Vaughn (1986) found that a solution of 0.003% pancreatin plus 0.002% EDTA in buffer was effective in removing Sf21 AE insect cells from plastic surfaces.

In the interest of producing cost-effectively abundant quantities of wild type baculoviruses such as AcNPV for bioinsecticide applications, the roller bottle method was proposed. Roller bottles are attractive because of the simpicity of the setup, the straightforward scale-up (multiple banks of roller bottles) and the possibility to contain episodic contamination in a single individual unit. In combination with semi-automatic systems dispensing medium and inoculum, roller bottles can be important contributors to the overall economy of scale: cell densities of 20 times the initial inoculum can be obtained and final cell concentrations equivalent to 1.3×10^7 $cells.ml^{-1}$ have been reached in 1750-cm^2 roller bottles holding 250 ml of growth medium (Weiss & Vaughn, 1986). Because of the tendency of insect cell lines to adhere less firmly than typical anchorage-dependent mammalian cell lines, roller bottle systems for insect cell propagation are made to revolve at slower rates than for mammalian cells, typically one revolution per 8 or 10 minutes (Vaughn, 1976; Weiss *et al.*, 1981).

For the anchorage-dependent Tn5 ("High Five") cells, prolific producers of secreted and post-translationally modified proteins, propagation at scales above 100 ml has been achieved by a combination of roller bottles and microcarriers (Wickham & Nemerow, 1993). While Tn5 cells in standard polystyrene roller bottles tended to attach partially and unevenly and to form large aggregates, they were able to attach much more reliably and to grow well when the roller bottles were precoated with DEAE-based microcarriers. An important issue for efficient recombinant protein production (Epstein-Barr viral attachment protein EBV gp105) was the optimization of initial (seeding) cell density and cell density at infection with the baculovirus vector (Wickham & Nemerow, 1993), since infection of *T. ni* above a critical cell density lowers the efficiency of baculovirus replication and hence heterologous gene expression (Wickham *et al.*, 1992).

A perfusion mode of cultivation can be used in combination with attached cell growth. Perfusion operation tends to maintain constant levels of nutrients and metabolic byproducts thus ensuring a relatively even cell growth environment, since spent culture medium is continuously replaced by fresh medium. A prototype attached growth system operating under perfusion, the Dyna Cell Propagator or "bulk culture vessel", was designed with the objective of increasing the available surface area of insect cell growth for production-scale application (Weiss & Vaughn, 1986). This system consists of three vessels connected in series which are modified roller bottles (volume: 1700 ml; growth surface: 9500 cm^2) equipped with a plastic (Melinex) spiral core each. In addition to a continuous perfusion system containing fresh and spent medium vessels and peristaltic pumps, the cell propagators were equipped with pH and DO monitoring capabilities. With a perfusion rate of 25 ml/hr the system operated at quasi-steady state for a month and allowed the continuous propagation of Sf21AE cells at about 10^7 $cells.ml^{-1}$ and the attainment of 2.3×10^7 $viral\ particles.ml^{-1}$ in the perfusate (Weiss & Vaughn, 1986).

The potential for scale-up of roller bottles, bulk culture vessels and similar surface growth-dependent



Aedes albopictus (mosquito) cells growing in the interior of collagen-based open-structured porous microspheres (Verax, NH).

Table 3. Examples of Insect Cell Bioreactors

Reactor Type	Scale	Remarks	References
Attached Growth			
Dyna Cell Propagator	1.7 L	Continuous perfusion	Weiss & Vaughn, 1986
Split-flow airlift	0.5 L	Glass bead packing	Chung *et al.*, 1993
Stirred tank reactor	2.0 L	Fibrous matrix spiral	Archambault *et al.*, 1994
Suspension Growth			
Airlift	21 L	Air bubble diameter control, serum-free medium	Maiorella *et al.*, 1988
Airlift	40 L	Serum-free medium	Weiss *et al.*, 1988
Airlift	60 L	Serum-free medium	Schlaeger *et al.*, 1994
Stirred Tank	11 L	Helical ribbon impeller	Kamen *et al.*, 1991
Stirred Tank	75 L	Hydrofoil impellers, directly sparged	Jain *et al.*, 1991 Junker *et al.*, 1994

systems becomes, nonetheless, problematic beyond a moderate level of production capacity, because of the fundamental limitation in surface upon further increase in reactor volume (surface-to-volume ratio decreases as volume increases). Attempts to overcome this inherent difficulty for attachment-dependent insect cell lines include the development of cultivation systems in which the cells are grown on support particles like microcarriers or glass beads. One such system for the cultivation of strain Tn5 used a packed bed column of 3-mm nonporous glass beads connected to a separate bubble column operating as a medium oxygenation device, so that the medium can circulate through the bed in a manner analogous to airlift systems (Shuler *et al.*, 1990). Because of the decoupling of aeration from the main bioreactor vessel, this system is potentially

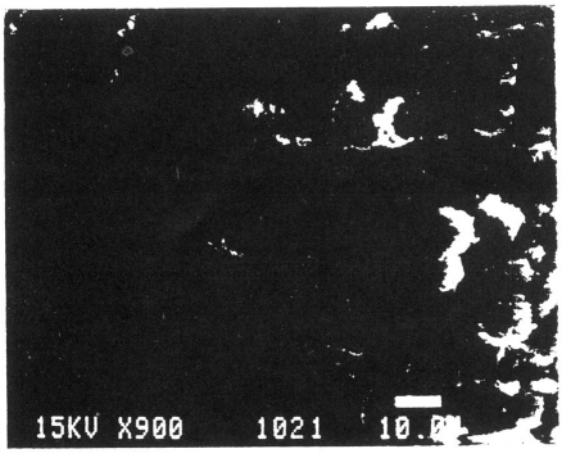


Figure 2. Trichoplusia ni cells (line Tn5 or "High Five") growing in and around open-structured non-woven fibrous polyester disks (Sterilin, UK).

scalable since oxygen supply and hydrodynamic shear do not have a strong dependence on bioreactor scale. Using this design, high cell densities and viabilities together with a production of about 200 mg/10^6 cells of recombinant ß-galactosidase was possible. In an improvement of this concept, the same group has used a split-flow, airlift reactor comprising a riser for bubble aeration and a downcomer where the glass beads or microcarriers colonized with Tn5 cells reside physically and are in constant contact with the circulating aerated medium, but are not directly exposed to sparging (Chung *et al.*, 1993). This split-flow airlift bioreactor does not require the use of pumps and has good potential for volumetric scale-up of processes utilizing cell lines which require a growth surface. In this application, the prototype laboratory system of 0.5 liters was used to demonstrate the production of a secreted, glycosylated recombinant protein, human secreted alkaline phosphate (seAP). With a ratio of riser to downcomer cross-sectional areas of 1, an aspect ratio of 4.4, an air flow rate of 54 ml/min in the riser and a packing of 2400 3-mm nonporous glass beads (found to be superior for Tn5 cell attachment to derivatized porous collagen Verax microspheres, Cytodex 1 or 3 microcarriers or 0.5-mm glass beads), a volumetric titer of 10.7 mg/ml of seAP was produced at a MOI of 10. On a specific production basis, the capacity of this novel airlift reactor system was similar to that of T-flasks at about 10.7 mg/10^6 cells, which may indicate that the intrinsic capacity of the strain to produce and export a complex recombinant protein is retained in a scalable system.

Immobilized insect cell reactors have also been reported in conjunction with different cell lines and supports, including the entrapment of Sf9 cells in alginate-polylysine microcapsules (King *et al.*, 1988), *Aedes albopictus* (mosquito) cells (Figure 1) in derivatized open-structure porous collagen microspheres (Verax, NH) applied as a suspension in stirred tank reactors (Agathos *et al.*, 1990), Sf9 cells in non-woven polyester support particles (Sterilin, UK) applied in a fixed bed reactor (Kompier *et al.*, 1991) and also Sf9 cells in a fibrous vertical cylinder mat wrapped around a rotating cage in a stirred reactor (Archambault *et al.*, 1994). In our own laboratory we have succeeded in cultivating Tn5 cells at densities of 9×10^7 cells.ml^{-1} (Figure 2) by using a 2.2-liter modified CelliGen Plus basket reactor (New Brunswick Scientific, Edison, NJ) packed with 0.6-cm polyester non-woven fabric support disks (Sterilin, UK). The polyester fibers are laminated onto a polypropylene "scaffold". The positively charged fabric surface allowed extensive growth of the Tn5 cell line. About 100 grams of supports were used per liter of packed bed and the surface available for growth was of the order of 100 cm^2 per cm^3 of bed volume. In a packed bed of this material the void fraction is 90% and only small pressure drops are encountered for medium circulation across the bed (packed rotating basket). The support is highly stable, steam-sterilizable and does not require any surface treatment to promote cell attachment.

The possibility of infecting immobilized insect cells with baculovirus at very high densities under optimized nutritional conditions augurs well for the potential of cell immobilization applications in production-level bioprocessing. A further description of insect cell immobilization is given elsewhere in this volume (Wu & Goosen, 1996).

Suspension growth systems

Among the recent demonstrations of scalable propagation of insect cells in suspension several involve the use of airlift devices (Maiorella *et al.*, 1988; Murhammer & Goochee, 1988;Weiss *et al.*, 1988; King *et al.*, 1992; Schlaeger *et al.*, 1994). Airlift reactors are reported to be simpler in design and construction than stirred tank reactors and, as a result, they lead to reduced capital and maintenance cost (no shaft bearings seals or drive mechanism to service) and reduced risk of microbial contamination, hence reliable and low-cost operation (Rhodes *et al.*, 1991). The design of concentric tube airlift reactors has the potential to provide adequate

oxygen transfer rates with low and homogeneously distributed shear compared to conventional stirred reactors or simple bubble columns. Aspect ratio and superficial gas velocity influence reactor performance and they must be assessed at each higher scale using as a criterion the meeting of desired oxygen transfer rates. The ratio of downcomer to riser cross sectional areas is critical to mixing and oxygen transfer in an airlift reactor, and as this ratio is increased the volumetric mass transfer coefficient (k_La) and the superficial velocity of the air decrease. The successful cultivation of Sf9 cells in a 21-liter airlift reactor for the production of macrophage colony stimulating factor was reported by Maiorella *et al.* (1988). Using a new serum-free medium formulation containing Pluronic F-68, plus a close control of the air bubble diameter (0.5-1.0 cm) to avoid coalescence, they optimized the process to a maximum cell density of 5.5×10^6 cells.ml^{-1} and a protein titer of 40 mg.l^{-1}, performances comparable to those obtained initially in 100-ml spinners. In a 14-liter airlift reactor operating at an aeration of 0.06 vvm with 1-cm air bubbles, a kinetic study of Sf9 growth and recombinant ß-galactosidase production showed that a very high cell density of 10^7 cells.ml^{-1} was possible and 0.33 mg protein per ml of serum-free medium (SF-900) was reached, i.e., about 37% of the level attained in shake flasks (King *et al.*, 1992). A bigger scale of cultivation proved possible when Weiss and colleagues (1988) used a microbial 40-liter concentric tube airlift fermentor to arrive at a cell density of 2.4×10^6 cells.ml^{-1} from an inocolum level of 2.0×10^5 cells.ml^{-1} over 11 days, while Schlaeger & co-workers (1994) adapted Tn5 cells to suspension growth in 60-liter airlifts in serum-free medium containing Pluronic F-68.

Conventional stirred tank systems have been used for large-scale insect cell culture ranging from volumes of 3 liters to over 150 liters (Weiss *et al.*, 1989; Jain *et al.*, 1991; Barkhem *et al.*, 1992; Guillame *et al.*, 1992; Bédart *et al.*, 1994b). The basic attraction of a stirred tank reactor system is its wide availability and suitability for multipurpose use (e.g., in a pilot plant where R&D tests may alternate among various microbial and animal cell systems for production of biologicals) and the long-standing experience in design and understanding of scale-up principles in microbial fermentation. The fear of hydrodynamic shear-induced damage to insect cells from air bubbles has motivated the development of bubble-free aeration techniques in stirred tanks which can be fitted with semipermeable membrane (silicon) tubing for oxygenation. Examples include the successful cultivation of *Mamestra brassicae* cells (Miltenburger & David, 1980) and Sf9 cells (Eberhard & Schügerl, 1987) in a 10-liter reactor encompassing 15 m of silicon tubing. Further improvements in oxygen transfer can be realized in this system when the aeration tubing is motorized with a reciprocating movement (Graf & Schügerl, 1991). Despite the demonstration of this system's feasibility at an intermediate scale, further scale-up will be limited by the progressively larger percentage of the reactor volume that will be occupied by the tubing, its high cost and the problems of membrane fouling anticipated. On the other hand, there is encouraging evidence that large-scale production of insect cells and their recombinant protein or viral products can be achieved with standard agitated reactors after relatively minor modification (design and configuration of impellers and aeration orifices, complementation of efficient surface aeration and non-aggressive sparging in combination with shear-protective media additives). For example, a modified 8-liter spinner reactor agitated with magnetic bars at 90 rpm and sparged directly with air at 75 ml.min^{-1} through a conventional ring sparger gave satisfactory cell densities ($2.9–3.9 \times 10^6$ cells.ml^{-1}) in serum-free medium (Weiss *et al.*, 1989). The same workers demonstrated the potential of a 3-liter spin-filter reactor to produce 10^6 units.ml^{-1} of recombinant human ß-interferon (Weiss *et al.*, 1989). In this design, the cells are physically segregated from the sparged medium through a rotating cage, continuous perfusion of the medium and removal of metabolic waste by-products is occurring and high cell densities of the order of 10^7 cells.ml^{-1} are attainable. In a variation of this technique, removal of spent medium is continuously effected through a microporous filtration module or a hollow fiber filtration cartridge, resulting in production of more than 11 viral polyhedra per cell, compared to only 9 without perfusion (Klöppinger *et al.*, 1990). The feasibility of high cell density scale-up was also demonstrated in a modified CelliGen (New Brunswick Scientific, Edison, NJ) bioreactor adapted for perfusion culture (Caron *et al.*, 1994). Sparging was done through a geotextile membrane on the base of the CelliGen's characteristic hollow impeller shaft; 0.5-mm bubbles were thus harmless for the cells that were not exposed to them. When perfused at 1 - 1.5 volume of medium per day, Sf9 cells grew from 4×10^6 to 15×10^6 cells.ml^{-1} over 3-4 days. The continuous perfusion was possible through prototype tangential filtration devices. A more complete discussion ot the potential of high cell density perfusion cultures is given elsewhere in this volume (Jäger, 1996). A compar-

ison of the performance of a prototype 11-liter stirred tank bioreactor equipped with a helical ribbon impeller (Kamen *et al.*, 1991) and a modified 4-liter stirred tank CelliGen bioreactor with two marine impellers and a special interfacial impeller (to improve surface aeration from the head space) used by the same group (Caron *et al.*, 1990) showed that the final Sf9 cell densities attained were comparable, in the vicinity of 5×10^6 cells.ml^{-1}. Differences in the yield of recombinant protein production (bovine rotavirus nucleocapsid protein VP6) were attributed to the use of different media between the two systems.

The lytic insect cell-baculovirus expression system imposes limitations on the useful length of a batch reactor cycle, hence continuous insect cell cultivation schemes have been proposed in order to reach high and uniform productivity. Cascades of multiple reactors have been reported (Kompier *et al.*, 1988; Klöppinger *et al.*, 1990; 1991; van Lier *et al.*, 1990; 1992; Zhang *et al.*, 1993). In these (semi-) continuous setups two or three reactors are run in series, the first for growth of uninfected cells followed by one or two vessels for cell infection and virus or protein production. The systems proposed by Klöppinger *et al.* (1990; 1991) for Sf9 cells and by Zhang *et al.* (1993) for Bm5 cells are in essence semi-continuous (repeated batch cultivation of cells and perfusion of medium in the infection reactor which can be infected several consecutive times) and achieve adequate and reproducible product titers. In contrast, the systems proposed by the Wageningen group (Kompier *et al.*, 1988; van Lier *et al.*, 1990; 1992) are completely continuous. The effective production period for polyhedra or ß-galactosidase in this scheme sems to be of the order of about 30 days, beyond which point there is an unrecoverable decline in productivity attributed to the "passage effect". This effect is considered to be due to the progressive enrichment of the serially passaged virus in defective interfering particles (DIP), i.e., deletion mutants interfering with the multiplication of fully functional polyhedrosis virus (Kool *et al.*, 1991; Wickham *et al.*, 1991). A concern to use low-passage viral inocula in continuous or semi-continuous bioprocessing schemes and the greater flexibility of fed-batch cultivation may improve the outlook of these modes of bioreactor operation.

Monitoring of insect cell growth and baculovirus infection phases

As in every bioprocessing scheme involving production-scale microbial or mammalian cell systems, the ultimate consistency of bioreactor-based insect cell cultivation can only be assured with adequate means of process monitoring and control in place. In addition to the traditional bioreactor cultivation parameters that are measurable on line on most vessels, such as temperature, pH and dissolved oxygen (DO), continual (ideally on-line) measurements of cell density and viability, of concentrations of substrates and byproducts, etc. are important for tracking the progress of the cultivation with time and for establishing simple empirical or model-based control actions in order to maintain optimal conditions or to steer the process in the direction most compatible with process goals. The choice of parameter to be monitored and controlled will be affected by the degree to which it influences, directly or indirectly, the efficiency of protein production in the insect cell-BEV system (see above). As with mammalian cell reactors, instrumentation of insect cell reactors can encompass both well-established technological tools for on- or off-line estimation of respiration rates and quotients (Kamen & Tom, 1994; Wong *et al.*, 1994; Hensler & Agathos, 1994) as well as less conventional accessories, such as optical density probes (Bédart *et al.*, 1994a) or coupling to biochemically relevant analyses of cell populations, such as flow cytometry (Al Rubeai *et al.*, 1994) or fluorescent probes (Hensler & Agathos, 1994).

The determination of viable and total cell count is among the most important measurements during both the growth and the production phase in insect cell reactors. Cell numbers are typically obtained off-line by counting trypan blue-stained and unstained portions of cell suspension aliquots in a hemocytometer or by using a Coulter™ particle counter. Although still widely practiced in laboratory and industrial situations, manual counting is labor-intensive and does not lend itself easily to automation, hence direct physical approaches are considerably more attractive for process data logging and control purposes. Measurements of optical density (OD) obtained either off-line on a spectrophotometer or on-line with an optical density sensor have been recently reported to be reliable means of assessing cell concentration in bioreactor-based insect cell cultures (Bédart *et al.*, 1994a). After establishing the validity of a simple relationship between OD and Sf9 insect cell density, Bédart *et al.* (1994a) recognized that the continuous monitoring of OD would be both useful and feasible in scale-up, since a new generation of steam sterilizable optical sensors have been introduced lately for on-line OD logging in animal bioreactors (Konstanti-

nov *et al.*, 1992; Zhou & Hu, 1994). The on-line OD measurement of cultured Sf9 insect cells was carried out with a MAX™ Cell Mass Sensor (Cerex Corporation, Ijamsville, MD) consisting of an optical probe and a data acquisition system connected to a personal computer. The fiber optic probe (5 mm light path) was equipped with a laser diode light source with a spectral range of 820-850 nm. The system also contained an integrated debubbler to minimize interference from air bubbles in the 20-liter bioreactor culture. The usefulness of OD monitoring is currently limited to the cell growth phase only, because it has been found that OD measurements in baculovirus-infected cultures grossly overestimate the actual cell density due to changes in cell size and to the presence of cell debris during the progress of infection (Bédart *et al.*, 1994a). However, with the high accuracies of on-line OD measurements for Sf9 cell densities up to 10^7 cells.ml^{-1} reported by these workers, it is easy to obtain satisfactory estimates of derivative (calculated) culture parameters, such as specific growth rates and specific nutrient consumption rates, which could be useful in their own right for process control.

Indirect measurement procedures linking cellular growth with aerobic respiration are also promising in insect cell bioreactors: for instance, Kamen & Tom (1994) have found that there is a consistent linear relationship between viable cell density and CO_2 Production Rate (CPR) during Sf9 cell growth over a wide range of cell concentration values ($0 - 10^8$ cells.ml^{-1}). Accurate on-line estimates of CPR and OUR are easy to obtain from instrumented bioreactors equipped with gateway offgas sensors such as a mass spectrometer (Kamen & Tom, 1994). Offgas analysis can therefore provide continuous accurate estimates of cell density during the insect cell growth phase. In addition, OUR in itself appears to be a useful metabolic parameter for tracking both growth and infection/protein production phases in insect cell bioreactors (Wong *et al.*, 1994; Hensler & Agathos, 1994). For exponentially growing uninfected Sf9 cells growing in a 1-liter stirred tank reactor it was found that OUR was proportional to cell density over a range of $1 \times 10^6 - 6 \times 10^6$ cells.ml^{-1} (Wong *et al.*, 1994). The respiratory quotient (RQ), defined as the ratio of CPR/OUR, has only recently been recognized as a potentially valuable indicator of the physiological state in animal cell culture (Bonarius *et al.*, 1995) and therefore it would be interesting to assess its utility in monitoring insect cell bioreactor cultivations (Kamen & Tom, 1994).

In contrast to the growth phase, the monitoring of baculovirus-infected cells is considerably more challenging, even though this phase is crucial for tracking and controlling protein or virus production. Conventionally, viable cell density in the phase of infection is followed in discrete samples by microscopic observation in combination with trypan blue dye exclusion, as mentioned above. In recent work we have evaluated the progress of infection of Sf9 cells in DO-controlled 250-ml stirred tanks for recombinant ß-galactosidase production by means of OUR and epifluorescence microscopy in addition to trypan blue staining (Hensler and Agathos, 1994). A combination live/dead fluorescent stain system (Molecular Probes, Eugene, OR) consisted of calcein AM and of ethidium homodimer stains. The calcein AM stain gave an assessment of cell viability by virtue of its hydrolysis into a highly fluorescing (green) product through the action of intracellular esterases, while ethidium homodimer provided a powerful visual evaluation of the status of infected cells since this stain penetrates compromised cellular membranes and intercalates with the cell's DNA producing a bright orange fluorescent glow. This combination live/dead staining procedure gave a better assessment of the gradual loss of viability of infected Sf9 cells compared to trypan blue exclusion, which tended to overestimate viability in the early stages of infection and to underestimate it 3-4 days post-infection. While this assessment was done by discrete sampling off-line, the samples required no special processing (fixing etc), hence fluorometric measurements can be potentially adapted to continuous monitoring on-line.

OUR has also been found useful in following the progress of the infection process given its transiently increasing profile (Hensler & Agathos, 1994) as well as the recently noted inverse correlation between the rate of rise in the specific OUR and the cell density at the time of infection (Wong *et al.*, 1994). Based on this observation Wong and co-workers suggest that OUR measurements can be used to monitor the efficiency of a batch infection process. Such a series of OUR measurements during the early stages of the infection may indicate whether the infection will proceed as predicted by comparing the culture OUR with the expected rate of specific OUR rise, and corrective interventions such as medium replenishment or nutrient feeding can then be imposed in order to restore the productivity, if the rate of increase in specific OUR is found to reflect a potentially low-yielding infection (Wong *et al.*, 1994).

Yet another useful means of monitoring baculovirus infection of cultured insect cells is flow cytometric

analysis of unprocessed culture samples (Al Rubeai *et al.*, 1994). In this work the orthogonal scattering properties were found to be linearly related to the percentage of infected Sf9 cells as assessed by optical microscopy. In addition, flow cytometric monitoring of cellular DNA identified the infectivity of the cells and the subsequent development of viral particles much earlier than visually possible.

The evaluation of optimal harvesting time is also a matter of careful consideration. It must be aided by reliable means of product monitoring and may well depend on the technique used. Hence methods of adequate continuous or discrete measurement of product formation in bioreactors are highly desirable. In our own work, gel electrophoretic, chromogenic (ONPG) or fluorometric methodologies for a model recombinant protein, bacterial ß-galactosidase, gave varying indications of peak gene expression for the same Sf9 culture (Hensler and Agathos, 1994). SDS-PAGE gel profiles indicated a peak in expression at 48-72 hours post infection, whereas the ONPG assay together with a fluorometric assay of unprocessed cells stained with fluorogenic ß-galactosidase substrates such as C_{12}FDG (ImaGene Green, Molecular Probes, Eugene, OR) indicated a peak later in the infection (96 hours). The power of visualization of cellular ß-galactosidase content through the same fluorometric methodology was also reported by others (Al Rubeai *et al.*, 1994).

Bioreactor mode of operation in relation to nutritional requirements: medium replenishment or nutrient addition strategies

We noted above that the desirability of maximal productivities by operating high cell density cultivations of insect cells must be tempered with the realization that nutritional control should be exercised in the infection phase, in order to overcome the apparent conflict between high-density infection and good recombinant protein formation.

During insect cell infection, cell division stops but other cell physiological activities continue, together with transcription and translation that is now commandeered towards viral multiplication and viral gene expression. Hence, for high virus or protein production to be accomplished, it is vital that the infected cell's machinery be kept under optimal physiological conditions, free of oxygen or nutritional limitations and of potential inhibitions from accumulating metabolic products. This realization has been supported by recent studies showing that by resuspending cells in fresh medium upon infection at high density and by maintaining aeration, specific productivity can be restored to that of low-density infection or even enhanced (Lindsay & Betenbaugh, 1992;Reuveny *et al.*, 1993a;Zhang *et al.*, 1993; 1994b; Hensler & Agathos, 1994). For example, an increase of Sf9 density at infection from 0.49×10^6 cells.ml^{-1} to 0.94×10^6 cells.ml^{-1} led to a 17 fold reduction in specific productivity of ß-galactosidase, yet resuspension in fresh medium *after* high-density infection restored specific productivity to 100% compared with the low-density culture and allowed a 26-fold increase in protein yield compared to infection in spent, unaerated medium (Lindsay & Betenbeugh, 1992). Similarly, by renewing the serum-free medium *before* infection at Sf9 cell densities of 2 and 3×10^6 cells.ml^{-1} a similar ß-galactosidase specific productivity was obtained to that observed at 0.5×10^6 cells.ml^{-1} in spent medium, whereas infection at 2×10^6 cells.ml^{-1} without complete medium renewal had previously led to a 3-fold decrease in protein per cell compared to that observed at 0.5×10^6 cells.ml^{-1} (Hensler & Agathos, 1994). In this case nutrient limitation was ascertained as the root cause of the unsatisfactory performance at high cell density infection and the medium renewal stategy led to a doubling of maximum volumetric productivity. In addition, measurements of nutrient specific consumption rates showed that the utilization of glucose and glutamine increased in infected Sf9 cells by 70% and 64% respectively, compared to the rates in exponentially growing uninfected cells (Hensler & Agathos, 1994). In a series of detailed nutritional studies, the limitations of recombinant protein (epoxide hydrolase) production in Sf9 cells by oxygen, glucose and glutamine were demonstrated and supplementation with these nutrients, together with DO control at 35% saturation, increased significantly the protein specific productivity at the high cell density of 4×10^6 cells.ml^{-1} (Wang *et al.*, 1993a, 1993b). Although no amino acid was found to be protein formation rate-limiting during the infection phase at the lower cell densities of $1.2 - 2.4 \times 10^6$ cells.ml^{-1} (Wang *et al.*, 1993b), there are indications that some amino acids may indeed be rate-limiting at high cell density infection, given the increases noted in their specific consumption rates after infection in comparison to their pre-infection values (Wong *et al.*, 1994). Along the same lines, direct introduction of fresh medium after viral infection increased productivity of recombinant HIV antigen CD4 at comparable cell densities (Lazarte *et al.*, 1992). In a study of recombinant protein (ß-galactosidase or glucocere-

brosidase) formation by Sf9 cells under oxygen sufficiency, protein production was tripled when the spent medium was replaced with fresh one before infection (Reuveny *et al.*, 1993a). In the same work, when the fresh medium was supplemented with either glucose alone or glucose, glutamine and yeastolate a 25% or a 100% increase in recombinant protein expression was observed, respectively.

Occasional reports of no direct effect of cell density levels nor of nutrient replacement strategies on protein productivity (Kioukia *et al.*, 1995; Zhang *et al.*, 1994b) may indicate that other simultaneous influences should also be examined for their role on the cells' infectivity and production potential at key points in the population's development. Notably, the difference in infectivity in different phases of the insect cells' growth curve may be due to the difference in cell cycle distribution between the G1, S and and G2/M phases and the higher susceptibility of S-phase cells to baculovirus replication (Kioukia *et al.*, 1995). Also if nutrient limitation is absent at the end of exponential growth, there is no advantage of medium replenishment for protein productivity (Zhang *et al.*, 1994b).

In sum, the decline of recombinant protein yield observed in cultures at late growth stage (high cell density) can be reversed partially or totally by one of the following stategic interventions: (a) by completely replacing spent medium with fresh before or after infection (Caron *et al.*, 1990; Lindsay & Betenbaugh, 1992; Hensler & Agathos, 1994), (b) by adding fresh medium directly to the infected sulture (Lazarte *et al.*, 1992) or (c) by simultaneous medium replacement and nutrient supplementation (Reuveny *et al.*, 1993a). Along similar lines, the promise of perfusion culture of insect cells for recombinant protein production at high cell densities (Caron *et al.*, 1994; Deutschmann & Jäger, 1994) is largely based on the continuous replacement op spent medium with fresh.

Feeding stategies designed for consistently high protein production should ideally be as simple as possible, especially if large volumes of mediuum are involved. The infection and post-infection stage of high density Sf9 cell culture has been studied recently with respect to optimal production of a heterologous protein by focusing on the dynamicss of the protein as it is being expressed. In a study of the production of a recombinant soluble immunoglobulin receptor (IgEr), the concentration of the protein increased for 4-5 days post-infection but it declined after day 6 (Kirn & Familetti, 1994). This group demonstrated that IgEr degradation could be minimized by addition of protein hydrolyzates early after baculovirus infection: by supplementing the serum-free ExCell 401 medium with 5 mg/ml meat hydrolyzate or casein hydrolyzate at 24 hours post infection, a 4-fold increase in IgEr was achieved over unsupplemented cultures or cultures supplemented prior to infection (Kirn & Familetti, 1994).

In a further recent development, Bédart & coworkers (1994b) have demonstrated that specific protein yields in normally low-producing late exponential phase cultures of Sf9 cells can be restored to high levels by the one-time addition of an ultrafiltrate of yeastolate and a concentrated mixture of amino acids; this can lead to high volumetric yields of ß-galactosidase galactosidase in the serum-free medium SF900II (Gibco/Life Technologies, Grand Island, NY), which supports among the highest Sf9 cell densities in batch culture (8×10^6 cells.ml^{-1}). The findings of the last two studies show that exhaustion of one or more nutrients rather than accumulation of inhibitory byproducts may be the main limiting factor in recombinant protein production by Sf9 cells in batch culture. From a process development point of view, this strategy simplifies large-scale protein production because it allows operations of growth and infection of high cell density without requiring a major step of cell-liquid separation for medium replacement.

Concluding Remarks

Despite the variety of bioreactor designs that have been studied and used in acedemic work and commercial R&D, no single system has proven so far to be universally superior in productivity and scalability. These are attractive features in most attached and suspension growth systems even though there is a tendency to favor the latter given their simplicity and the long-standing experience associated with them from microbial fermentation. The recent insights concerning the culturability of insect cells even under conditions of direct aeration in stirred-tank and airlift reactors and the rationalization of the influences of oxygen, other nutrients, cell density, multiplicity of infection, etc. on productivity, should help demystify insect cell culture in bioreactors. It is especially encouraging that microbial pilot plant reactors of 75 and 280 liters can be retrofitted in terms of temperature and agitation control, impeller type (Rushton turbines of hydrofoils), versatile aeration capability through both head-space and open-pipe sparging, for successful cultivation of

insect and mammalian cells in a rapid and inexpensive manner (Junker *et al*., 1994). Thus it may well be that large-scale insect cell culture technology will be progressively practiced through the use of reactors flexibly designed for both microbial and animal cell cultivation. Nonetheless, reactor designs for attached or immobilized insect cell growth (including cell retention devices for perfusion culture) may also be appealing alternatives for processes where the scale-up of production capacity may revolve around very stringent requirements of the particular cell line (attachment dependence, very high densities on or in particular support matrices, protection against physical stresses, ease of medium exchange, etc). The future of bioprocessing with insect cells can be anything but bleak given the present wealth of options in both equipment and operation stategy.

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References

Agathos SN(1993) The future of cell culture engineering. In: Goosen MFA, Daugulis AJ & Faulkner P (eds) Insect Cell Culture Engineering, pp. 221–240. Marcel Dekker, New York.

Agathos SN (1994) Large scale insect cell production. In: Maramorosch K & McIntosh AH (eds) Insect Cell Biotechnology, pp. 89–103. CRC Press, Roca Raton.

Agathos SN, Jeong Y-H & Venkat K (1990) Growth kinetics of free and immobilized insect cell cultures. Ann. N.Y. Acad. Sci. 589: 372–398.

Al Rubeai M, Kioukia N & Emery AN (1994) Monitoring of insect-baculovirus culture processes by flow cytometry. Presented at Cell Culture Engineering IV, Engineering Foundation Conferences, San Diego, CA 7-12 March 1994 (Abstract WE3, Cell Culture Engineering IV Program and Abstracts, p. 86).

Archambault J, Robert J & Tom RL (1994) Culture of immobilized insect cells. Bioprocess Eng. 11: 189–197.

Barkhem T, Carlsson B, Danielsson A, Norinder U, Frieberg H & Öhman L (1992) Production in a 100 liter stirred tank reactor of functional full length human thyroid receptor b1 in Sf-9 insect cells using a recombinant baculovirus. In: Vlak JM, Schlaeger E-J & Bernard AR (eds) Baculovirus and Recombinant Protein Production Processes, pp. 235–246. Editiones Roche, Basel.

Bédart C, Jolicoeur M, Jardin B, Tom R, Serret S & Kamen A (1994a) Insect cell density in bioreactor cultures can be estimated from on-line measurements of optical density. Biotechnol. Techniques 8: 605–610.

Bédart C, Kamen A, Tom R & Massie B (1994b) Maximization of recombinant protein yield in the insect cell/baculovirus system by one-time addition of nutrients to high-density batch cultures. Cytotechnology 15: 129–138.

Bonarius HPJ, de Gooijer CD, Tramper J & Schmid G (1995) Determination of the respiration quotient in mammalian cell culture in bicarbonate buffered media. Biotechnol. Bioeng. 45: 524–535.

Caron AW, Archambault J & Massie B (1990) High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. Biotechnol. Bioeng. 36: 1133–1140.

Caron AW, Tom RL, Kamen AA & Massie B (1994) Baculovirus expression system scaleup by perfusion of high-density Sf-9 cell cultures. Biotechnol. Bioeng. 43: 881–891.

Chalmers JJ (1994) Cells and bubbles in sparged bioreactors. Cytotechnology 15: 311–320.

Chung IS, Taticek RA & Shuler ML (1993) Production of human alkaline phosphatase, a secreted glycosylated protein, from a baculovirus expression system and the attachment-dependent cell line *Trichoplusia ni* BTI-Tn 5B1-4 using a split-flow, airlift bioreactor. Biotechnol. Progress 9: 675–678.

de Gooijer CD, van Lier FLJ, van den End EJ, Vlak JM & Tramper J (1989) A model for baculovirus production with continuous insect cell cultures. Appl. Microbiol. Biotechnol. 30: 497–501.

Depinto W & Familetti PC (1994) Increased expression of recombinant proteins from baculovirus infected cultures using Tn-5B1-4 (High Five) insect cells. In Vitro 30: 722 (Presented at the 1994 Cell and Tissue Culture Congress, Research Triangle Park, NC, 4-7 June 1994).

Deutschmann SM & Jäger V (1994) Optimization of the growth conditions of Sf-21 insect cells for high-density perfusion culture in stirred-tank bioreactors. Enzyme Microb. Technol. 16: 1133–1140.

Eberhard U & Schügerl K (1987) Investigations of reactors for insect cell culture. Develop. Biol. Standard. 66: 325–330.

Fleischaker RJ & Sinskey AJ (1981) Oxygen demand and supply in cell culture. Eur. J. Appl. Microbiol. Biotechnol. 12: 193–197.

Goldblum S, Bae Y-K, Hink WF & Chalmers J (1990) Protective effect of methylcellulose and other polymers on insect cells subjected to laminar shear stress. Biotechnol. Progress 6: 383–390.

Graf H & Schügerl K (1991) Influence of the reciprocating movement on the performance of a membrane aeration system in insect cell cultures. Biotechnol. Techniques 5: 91–94.

Guillame JM, Soria HM, Couteault N, Hurwitz DR, Multon MC & Crespo A (1992) Insect cell fermentation scale-up for recombinant protein production. In: Vlak JM, Schlaeger E-J & Bernard AR (eds) Baculovirus and Recombinant Protein Production Processes, pp. 255–261. Editiones Roche, Basel.

Hensler WT & Agathos SN (1994) Evaluation of monitoring approaches and effects of culture conditions on recombinant protein production in baculovirus-infected insect cells. Cytotechnology 15: 177–186.

Hensler W, Singh V & Agathos SN (1994) Sf9 insect cell growth and **β-galactosidase** production in serum and serum-free media. Ann. N.Y. Acad. Sci. 745: 149–166.

Hink WF (1982) Production of *Autographa californica* nuclear polyhedrosis virus in cells from large-scale suspension cultures. In: Kurstak E (ed.) Microbial and Viral Pesticides, pp. 493–506. Marcel Dekker, New York.

Hink WF & Strauss EM (1976) Growth of *Trichoplusia ni* (TN-368) cells in suspension culture. In: Kurstak E & Maramorosch K (eds) Invertebrate Tissue Culture, pp. 297–300. Academic Press, New York.

Hink WF & Strauss EM (1980) Semi-continuous culture of the TN-368 cell line in fermenters with virus production in harvested cells. In: Kurstak E, Maramorosch K & Dübendorfer A (eds) Invertebrate Systems In Vitro, pp 27–33. Elsevier-North Holland Biomedical Press, Amserdam.

Jäger V (1996) Perfusion culture of insect cells. This volume.

Jain D, Ramasubramanyan K, Could S, Lenny A, Candelore M, Tota M, Strader C, Alves K, Cuca G, Tung JS, Hunt G, Junker B, Buckland BC & Silberklang M (1991) Large-scale recombinant protein production using the insect cell-baculovirus expression vector system: antistasin and β-adrenergic receptor. In: Spier RE, Griffiths JB & Meignier B (eds.) Production of Biologicals from Animal Cells in Culture, pp. 345–350. Butterworth-Heinemann, Oxford.

Junker BH, Hunt G, Burgess B, Aunins J & Buckland BC (1994) Modified microbial fermenter performance in animal cell culture and its implications for flexible fermenter design. Bioprocess Eng. 11:57–63.

Kamen AA, Tom RL, Caron AW, Chavarie C, Massie B & Archambault J (1991) Culture of insect cells in a helical ribbon impeller bioreactor. Biotechnol. Bioeng. 38: 619–628.

Kamen AA & Tom R (1994) Mass spectroscopy determination of insect cell respiration rates in culture and production processes. In: Spier RE, Griffiths JB & Berthold W (eds) Animal Cell Technology. Products of Today, Prospects for Tomorrow (12th Meeting of the European Society for Animal Cell Culture Technology) pp. 345–350. Butterworth-Heinemann, Oxford.

Kim K & Familetti PC (1994) Optimal culture conditions for the production of recombinant proteins from baculovirus infected cells. In Vitro 30: 722 (Presented at the 1994 Cell and Tissue Culture Congress, Research Triangle Park, NC, 4-7 June 1994).

King GA, Daugulis AJ, Faulkner P, Bayly D & Goosen MFA (1988) Growth of baculovirus-infected insect cells in microcapsules to a high cell and virus density. Biotechnol. Lett 10: 683–688.

King G, Kuzio J, Daugulis A, Faulkner P, Alien B, Wu J & Goosen MFA (1991) Assessment of virus production and chloramphenicol acetyl transferase expression by insect cells in serum-free and serum-supplemented media using a temperature-sensitive baculovirus. Biotechnol. Bioeng. 38: 1091–1099.

King GA, Daugulis AJ, Faulkner P & Goosen MFA (1992) Recombinant ß-galactosidase production in serum-free medium in a 14-L airlift bioreactor. Biotechnol. Progr. 8: 567–571.

Kioukia N, Nienow AW, Emery AN & Al-Rubeai M (1995) Physiological and environmental factors affecting the growth of insect cells and infection with baculovirus. J. Biotechnol. 38: 243–251.

Klöppinger M, Fertig G, Fraune E & Miltenburger HG (1990) Multistage production of *Autographa california* nuclear polyhedrosis virus in insect cell cultures. Cytotechnology 4: 271–278.

K16ppinger M, Fertig G, Fraune E & Miltenburger HG (1991) High cell density perfusion culture of insect cells for production of baculovirus and recombinant protein. In: Spier RE, Griffiths JB & Meignier B (eds) Production of Biologicals from Animal Cells in Culture (10th Meeting of the European Society for Animal Cell Culture Technology) pp. 470–476. Butterworth-Heinemann, Oxford.

Kompier R, Tramper J & Vlak JM (1988) A continuous process for production of baculovirus using insect-cell cultures. Biotechnol. Lett. 10: 849–854.

Kompier R, Kislev N, Segal I & Kadouri A (1991) Use of a stationary bed reactor and serum-free medium for the production of recombinant protein in insect cells. Enzyme Microb. Technol. 13: 822–827.

Konstantinov KB, Pambayun R, Matanguihan R, Yoshida T, Perusich CM & Hu W-S (1992) On-line monitoring of hybridoma cell growth using a laser turbidity sensor. Biotechnol. Bioeng. 40: 1337–1342.

Kool M, Vondken JW, van Lier FLJ, Tramper J & Vlak JM (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology 183: 739–746.

Lazarte EJ, Tossin P-F & Nicolau C (1992) Optimization of the production of full-length rCD4 in baculovirus-infected Sf9 cells. Biotechnol. Bioeng. 40: 214–217.

Licari P & Bailey JE (1991) Factors influencing recombinant protein yields in an insect cell – baculovirus system: multiplicity of infection and intracellular protein degradation. Biotechnol. Bioeng. 37: 238–246.

Licari P & Bailey JE (1992) Modeling the population dynamics of baculovirus-infected insect cells: optimizing infection stategies for enhanced recombinant protein yields. Biotechnol. Bioeng. 39: 432–441.

Lindsay DA & Betenbaugh MJ (1992) Quantification of cell culture factors affecting recombinant protein yields in baculovirus-infected insect cells. Biotechnol. Bioeng. 39: 614–618.

Maiorella B, Inlow D, Shauger A & Harano D (1988) Large-scale insect cell culture for recombinant protein production. Bio/Technol. 6: 1406–1410.

Miltenburger HG & David P (1980) Mass production of insect cells in suspension. Devel. Biol. Standard. 46: 183–186.

Murhammer DW & Goochee CF (1988) Scaleup of insect cell cultures: protective effects of Pluronic F-68. Bio/Technol. 6: 1411–1418.

Neutra R, Levi B-Z & Shoham Y (1992) Optimization of protein production by the baculovirus expression vector system in shake flasks. Appl. Microbiol. Biotechnol. 37: 74–78.

Ogonah O, Shuler ML & Granados RR (1991) Protein production (ß-galactosidase) from a baculovirus vector in *Spodoptera frugiperda* and *Trichoplusia ni* cells in suspension culture. Biotechnol. Lett. 13: 265–270.

Papoutsakis ET (1991) Media additives for protecting freely suspended animal cells against agitation and aeration damage. Trends Biotechnol. 9: 316–324.

Reuveny S, Kemp CW, Eppstein L & Shiloach J (1992) Carbohydrate metabolism in insect cell cultures during cell growth and recombinant protein production. Ann. N.Y. Acad. Sci. 665: 220–237.

Reuveny S, Kirn YJ, Kemp CW & Shiloach J (1993a) Production of recombinant proteins in high-density insect cell cultures. Biotechnol. Bioeng. 42: 235–239.

Reuveny S, Kim YJ, Kemp CW & Shiloach J (1993b) Effect of temperature and oxygen on cell growth and recombinant protein production in insect cell cultures. Appl. Microbiol. Biotechnol. 38: 619–623.

Rhodes M, Gardiner S & Broad D (1991) Scaleup of animal cell suspension culture. In: Ho CS and Wang DIC (eds.) Animal Cell Bioreactors, pp. 253–268. Butterworth-Heinemann, Boston.

Schlaeger E-J, Foggetta M, Vonach JM & Christensen K (1993) SF-1, a low cost culture medium for the production of recombinant proteins in baculovirus infected insect cells. Biotechnol. Techniques 7: 183–188.

Schlaeger E-J, Wippler J & Foggetta M (1994) Scale-up of baculovirus expressed recombinant proteins using the High Five insect cell line. Presented at Cell Culture Engineering IV, Engineering Foundation Conferences, San Diego, CA 7-12 March 1994 (Abstract WE2, Cell Culture Engineering IV Program and Abstracts, p. 85).

Schopf B, Howaldt MW & Bailey JE (1990) DNA distribution and respiratory activity of *Spodoptera frugiperda* populations infected with wild-type and recombinant *Autographa California* nuclear polyhedrosis virus. J. Biotechnol. 15: 169–186.

Scott RI, Blanchard JH & Ferguson CHR (1992) Effects of oxygen on recombinant protein production by suspension cultures of Sf9 insect cells. Enzyme Microb. Technol. 14: 798–804.

Shuler ML, Cho T, Wickham T, Ogonah O, Kool M, Hammer DA, Granados RR & Wood HA (1990) Bioreactor development for production of viral pesticides or heterologous proteins in insect cell cultures. Ann. N.Y. Acad. Sci. 589: 399–422.

Streett DA & Hink WF(1978) Oxygen consumption of TN 368 cell line infected with AcNPV. J. Invertebr. Pathol. 31: 112–122.

Tramper J, Williams JB, Joustra D & Vlak JM (1986) Shear sensitivity of insect cells in suspension. Enzyme Microb. Technol. 8: 33–36.

Tramper J, de Gooijer KD & Vlak JM (1993) Scale-up considerations and bioreactor development for animal cell cultivation. In: Goosen MFA, Daugulis AJ & Faulkner P (eds) Insect Cell Culture Engineering, pp. 139–177. Marcel Dekker, New York.

van Lier FLJ, van den End EJ, de Gooijer CD, Vlak JM & Tramper J (1990) Continuous production of baculovirus in a cascade of insect-cell reactors. Appl. Microbiol. Biotechnol. 33: 43–47.

van Lier FLJ, van der Meijs WCJ, Grobben NA, Olie RA, Vlak JM & Tramper J (1992) Continuous ß-galactosidase productionwith a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. 22: 291–298.

Vaughn JL (1976) The production of nuclear polyhedrosis virus in large-volume cell cultures. J. Invertebr. Pathol. 28: 233–237.

Wang M-Y, Kwong S & Bentley WE (1993a) Effects of oxygen/glucose/glutamine feeding in insect cell baculovirus protein expression: a study on epoxide hydrolase production. Biotechnol. Progr. 9: 355–371.

Wang M-Y, Vakharia V & Bentley WE (1993b) Expresion of epoxide hydrolase in insect cells: a focus on the infected cell. Biotechnol. Bioeng. 42: 240–246.

Wang MY, Valler M, Pulliam TR & Bentley WE (1995) Elicitation of protease activity in *Spodoptera frugiperda* cell cultures. Presented at the 209th American Chemical Society National Meeting, Anaheim, CA 2-6 April 1995 (Abstract BIOT 120).

Weiss SA & Vaughn JL (1986) Cell culture methods for large-scale propagation of baculoviruses. In: Granados RR and Frederici BA (eds) The Biology of Baculoviruses, Vol. 2 Practical Applications for Insect Control, pp. 64–87. CRC Press, Boca Raton.

Weiss SA, Smith GC, Kalter SS & Vaughn JL (1981) Improved method for the production of insect cell cultures in large volume. In Vitro 17: 495–502.

Weiss SA, Orr T, Smith GC, Kalter SS, Vaughn JL & Dougherty EM (1982) Quantitative measurement of oxygen consumption in insect cell culture infected with polyhedrosis virus. Biotechnol. Bioeng. 24: 1145–1154.

Weiss SA, DeGiovanni A, Godwin G & Kohler J (1988) Large scale cultivation of insect cells. In: Roberts DW and Granados RR (eds) Conference on Biotechnology Biological Pesticides and Novel Plant-Pest Resistance for Insect Pest Management, Boyce Thompson Institute for Plant Research, Cornell University, July 18-20, 1988, pp. 22–30 Cornell University Press, Ithaca, NY.

Weiss SA, Belisle BW, DeGiovanni A, Godwin G, Kohler J & Summers MD (1989) Continuous cell lines as substrates for biologicals. Devel. Biol. Standard. 70: 272–279.

Wickham TJ, Davis T, Granados RR, Hammer DA. Shuler ML & Wood HA (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnol. Lett 13: 483–488.

Wickham TJ, Davis T, Granados RR, Shuler ML & Wood HA (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol. Progress 8: 391–396.

Wickham TJ & Nemerow GR (1993) Optimization of growth methods and recombinant protein production in BTI-TN-5B1-4 insect cells using the baculovirus expression vector. Biotechnol. Progress 9: 25–30.

Wong TKK, Nielsen LK, Greenfield PF & Reid S (1994) Relationship between oxygen uptake rate and time of infection of Sf9 insect cells infected with a recombinant baculovirus. Cytotechnology 15: 157–167.

Wood HA, Johnston LB & Burand JP (1982) Inhibition of *Autographa californica* nuclear polyhedrosis virus replication in high-density *Trichoplusia ni* cell cultures. Virology 119: 245–254.

Wu J, King G, Daugulis AJ, Faulkner P, Bone DH & Goosen, MFA (1989) Engineering aspects of insect cell suspension culture: a review. Appl. Microbiol. Biotechnol. 32: 249–255.

Wu JY & Goosen MFA (1996) Insect cell immobilization. This volume.

Zhang J, Kalogerakis N, Behie LA & latrou K (1993) A two-stage bioreactor system for the production of recombinant proteins using a genetically engineered baculovirus/insect cell system. Biotechnol. Bioeng. 42: 357–366.

Zhang J, Kalogerakis N & Behie LA (1994a) Optimization of the physicochemical parameters for the culture of *Bombyx mori* insect cells used in recombinant protein production. J. Biotechnol. 33: 249–258.

Zhang J, Kalogerakis N, Behie LA & latrou K (1994b) Optimum infection conditions for recombinant protein production in insect cell (Bm5) suspension culture. Biotechnol. Progr. 10: 636–643.

Zhou W & Hu W-S (1994) On-line characterization of a hybridoma cell culture. Biotechnol. Bioeng. 44: 170–177.

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Perfusion bioreactors for the production of recombinant proteins in insect cells

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Key words: insect cell culture, perfusion culture, membrane perfusion, crossflow microfiltration, baculovirus, bioreactor, fluidized bed, packed bed, recombinant protein production

Introduction

One of the fundamental reasons for using perfusion for insect cell culture is to reach higher cell densities which result in higher product titers and volumetric productivities. The growth of cells in customary insect cell culture media is usually limited to a maximum cell density of 3 to 6×10^6 cells ml^{-1}.However, cell densities of more than 1×10^7 cells ml^{-1} can be obtained by optimizing these media formulations by simple addition of a few nurients such as glucose and glutamine (Ackermann *et al.,* 1994). Cell densities of up to 3×10^7 cells ml^{-1} were achieved using more sophisticated feeding strategies (Bédard *et al.,* 1995). In contrast to mammalian cells there appears to be no critical accumulation of toxic metabolic products at these cell densities. Therefore, one of the most important advantages of continuous perfusion culture, namely the removal of cytotoxic metabolites such as ammonium, appears to be irrelevant to insect cell culture.

Typical problems of perfusion culture include the more complex bioreactor setup and in the case of cells requiring Baculovirus infection with baculovirus an increasingly unfavourable ratio of virus inoculum to the total reactor volume. Furthermore, the cell specific productivity of *Trichoplusia ni* cells can be drastically reduced at higher cell densities. This 'cell density effect' has been observed with Tn 368 cells (Wood *et al.,* 1982) as well as with BTI-Tn 5B1–4 cells (Chung & Shuler, 1993). Another problem is the rapidly declining production of infectious virus particles with increasing number of passages, the so-called 'passage effect' which is encountered during continuous production processes (Kompier *et al.,* 1988; Tramper *et al.,* 1990; van Lier *et al.,* 1990). As expected, recombinant protein production is also reduced by this effect (van Lier *et al.,* 1992, 1994). However, when the production of large amounts of recombinant protein requires multiple virus passages this phenomenon occurs regardless of the use of perfusion culture or a cascade of batch reactors.

On the other hand, the use of perfusion culture in insect cell cultivation and production of recombinant proteins features numerous advantages. In addition to the possible increase in cell specific and volumetric productivity, the residence time of secreted recombinant proteins in the bioreactor is significantly reduced, thus minimizing product degradation. By using microfiltration systems, the first step of downstream processing, the separation of a secreted recombinant protein from cell debris is already integrated into the production process. Furthermore, because of the continuous supply of culture medium which prevents cell death due to lack of nutrients, the production process is virtually independent of the speed of infection. This enables the use of an extremely low multiplicity of infection for the production of secreted proteins.

Internal membrane perfusion

Continuous medium exchange by internal membrane perfusion does not require additional pumping of cells through a filtration system. Therefore, cells are not

exposed to any additional shear stress. Fraune *et al.* (1991) have used a static PTFE hollow fiber membrane module for the perfusion culture of various animal cell lines including *Spodoptera frugiperda* but no further details are given in their paper. An identical module has been used by Klöppinger *et al.* (1990, 1991) for the cultivation of Sf9 cells in a two stage process. A maximum cell density of 1×10^7 ml^{-1} was achieved in a silicone membrane-aerated 1.5-liter stirred tank reactor for the production of wild-type *Autographa californica* MNPV or recombinant β-galactosidase. A different bioreactor system with membrane aeration via microporous polypropylene tubings and an additional hydrophilized polypropylene membrane for perfusion, both mounted on a tumbling stirrer has been used for the cultivation of IPLB-SF–21 AE cells. Cell densities of 2×10^7 ml^{-1} (Deutschmann & Jäger, 1991) or 5.5×10^7 ml^{-1} (Deutschmann & Jäger, 1994) were reached in a 1.2-liter stirred tank reactor with perfusion rates of 3 and 4 reactor volumes per day, respectively. Infection with recombinant Baculovirus and consequent recombinant protein production was performed at a cell density of up to 2×10^7 ml^{-1} (Jäger *et al.,* 1992) in bioreactors with 1.4 to 6 liter of working volume. Klöppinger *et al.* (1991) used a relatively high multiplicity of infection (MOI) of 5 to 10, which presupposes high titered virus stocks or large volumes of virus suspension for infection. Jäger *et al.* (1992) used an extremely low MOI of 0.015, which requires an almost complete retention of virus within the bioreactor in order to facilitate efficiently secondary infection of all noninfected cells irrespective of the perfusion rate. A retention of more than 99.9% was achieved despite of the fact that the membrane pore size of 0.2 μm should theoretically allow a complete penetration of the virus. More recently, an even lower MOI of 0.001 has been used successfully for recombinant protein production with this perfusion system (Jäger, unpublished results) (Figure 1). Schütz *et al.* (1991) cultivated the *Drosophila* cell lines Kc and Schneider–2, which have been used for stable transfection, in a 1.4-liter bioreactor of the same type resulting in maximum cell densities of 1.7×10^7 ml^{-1} and 3.4×10^7 ml^{-1}, respectively. Data of bioreactor cultivations with internal membrane perfusion are summarized in Table 1. The most significant drawback of internal membrane perfusion is the limited potential for scale-up. However, a scale-up to more than 200 liters of working volume appears to be feasible.

External membrane perfusion

In contrast to the special reactor configuration required for internal membrane perfusion almost any type of stirred tank or airlift bioreactor can be combined with external membrane perfusion provided that the reactor system has sufficient capacity to aerate higher cell densities without increasing shear forces (introduced by vigorous mixing or sparging) to a critical extent. In addition, external membrane perfusion offers the advantage that the microfiltration module can be exchanged if the need arises from membrane blocking. However, pumping of cells and circulation through crossflow microfiltration systems is able to generate substantial laminar shear stress to insect cells (Maiorella *et al.,* 1991). Therefore, choice of the crossflow system and the circulation pump as well as adjustment of flow rates are of particular importance.

First attempts to use external membrane modules for medium exchange were based on a two stage system for the production of wild-type virus (Klöppinger *et al.,* 1990). Propagation of Sf9 cells as well as virus propagation were performed in silicone membrane-aerated 1.5-liter stirred tank bioreactors. The reactor used for virus propagation was perfused by means of an internal microporous tubing system with 1 μm pore size, whereas the reactor for cell propagation was equipped with an external Microgon MiniKros hollow fiber crossflow filtration system. However, this system was not used continuously but in a batchwise mode to allow medium exchange prior to infection.

Continuous perfusion in a 4-liter Celligen reactor combined with a home- designed tangential flow filtration system (de la Broise *et al.,* 1991) resulted in densities of 1.5×10^7 Sf9 cells ml^{-1} (Massie *et al.,* 1992). However, the product yield, obtained when cells were infected at 1.2×10^7 ml^{-1} was reduced to 50–75% when compared to batch culture. This process was further optimized by introduction of different spiral-like tangential flow systems with or without integrated membrane aeration (Caron *et al.,* 1994). Perfusion at rates between 1 and 4 reactor volumes per day was used in the cell propagation phase as well as in the recombinant protein production phase. 45 minutes after infection the tangential flow system was also used to concentrate cells before the initial culture volume was reconstituted. Again, a reduced product yield of 75% compared to spinner flasks was observed. In contrast, Cavegn & Bernard (1992) obtained a normalized yield varying from 0.5–3.5 compared to batch culture and a significantly increased cell specific productivity (1.5–8

Table 1. Comparison of perfusion processes using internal membrane filtration systems

Reactor type	Working volume	Aeration	Perfusion system	Cell line	Max. cell density/ ml^{-1}	Max. perfusion rate/d^{-1}	Max. cell density during infection/ml^{-1}	MOI	Culture medium	References
Stirred tank	1.5 liters	static silicone membrane	static PTFE-hollow fiber	Sf9	1×10^7	?	1×10^7	1	TC100 +FBS	Klöppinger *et al.*, 1991
Stirred tank	1.4 liters	polypropylene membrane	polypropylene membrane	IPLB-SF–21 AE	2×10^7	3	–	–	IPL–41/TPB +FBS	Deutschmann & Jäger, 1991
Stirred tank	1.4 liters	polypropylene membrane	polypropylene membrane	IPLB-SF–21 AE	5.5×10^7	4	–	–	IPL–41/TPB +FBS	Deutschmann & Jäger, 1994
Stirred tank	2.5 liters	polypropylene membrane	polypropylene membrane	IPLB-SF–21 AE	5.5×10^7	4	2×10^7	0.015	IPL–41/TPB+FBS or Excell 401	Jäger *et al.*, 1992
Stirred tank	1.4 liters	polypropylene membrane	polypropylene membrane	Schneider–2	3.4×10^7	1.9	–	–	M3+FBS+Suppl.	Schütz *et al.*, 1991
Stirred tank	1.4 liters	polypropylene membrane	polypropylene membrane	Kc	1.7×10^7	0.9	–	–	D20	Schütz *et al.*, 1991

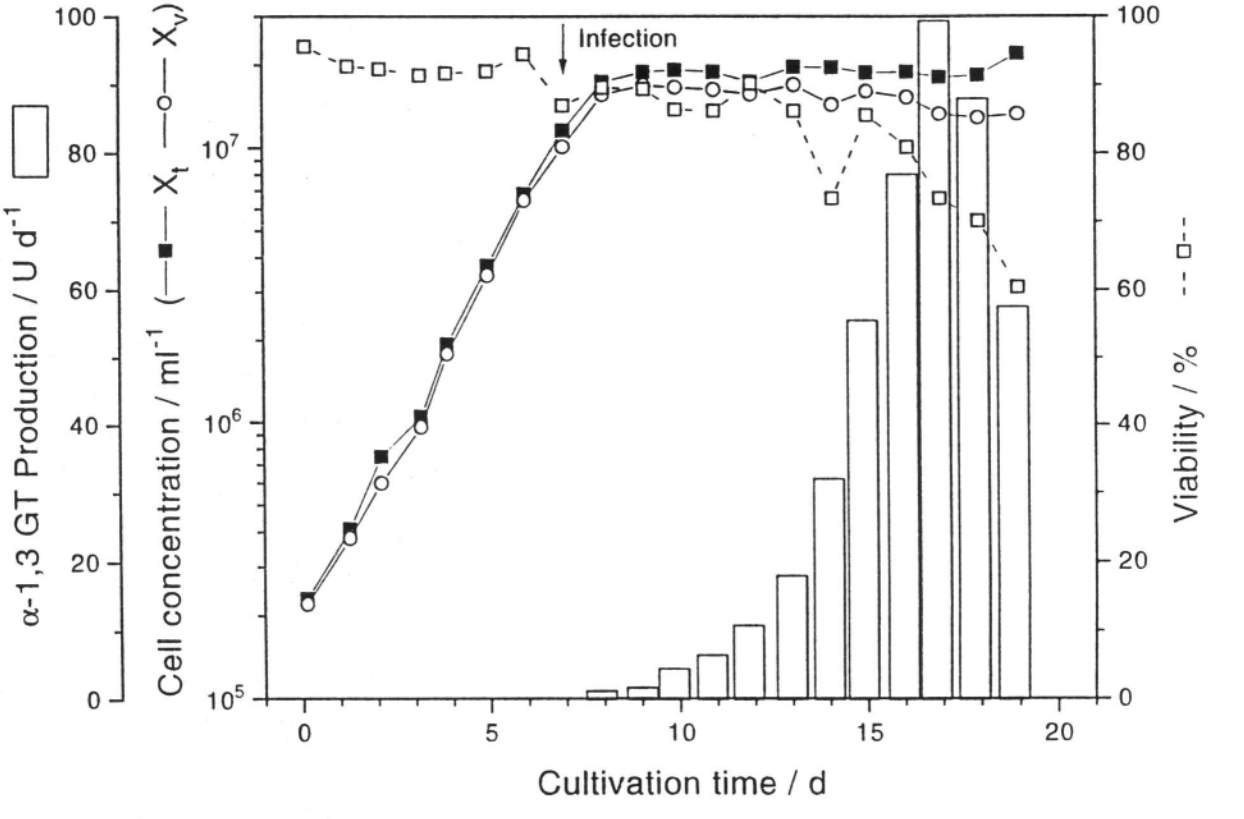

Figure 1. Cultivation of IPLB-SF–21 AE cells in a 2.5-liter stirred tank perfusion reactor for the production of α–1,3 galactosyltransferase (Jäger, unpublished results). Medium perfusion was started at day 6 at a cell density 7×10^6 ml^{-1}. Cells were infected at a cell density of 1×10^7 ml^{-1} with an extremely low multiplicity of infection of 0.001. Accordingly, maximum product formation was observed only 10 days post infection but at a cell specific productivity comparable to optimized batch culture.

Table 2. Comparison of perfusion processes using external membrane filtration systems

Reactor type	Working volume	Aeration	Perfusion system	Cell line	Max. cell density/ ml^{-1}	Max. perfusion rate/d^{-1}	Max. cell density during infection /ml^{-1}	MOI	Culture medium	References
Celligen	4 liters	caged sparging	flat membrane zig-zag channel	Sf9	1.5×10^7	?	1.2×10^7	?	TNM-FH +FBS	Massie *et al.*, 1992
Celligen	4 liters	caged sparging	flat membrane spiral-like channel	Sf9	1.5×10^7	4	1.4×10^7	2–4 +FBS	TNM-FH	Caron *et al.*, 1994
Stirred tank	4 liters	pulse sparging	hollow fiber	Sf9	3.0×10^7	5	1.1×10^7	5	Excell 400	Cavegn & Bernard, 1992 Cavegn *et al.*, 1992
Stirred tank	5 liters	direct sparging	hollow fiber	Sf9	1.0×10^7	0.6	–	–	ICF-WB	Reuveny *et al.*, 1994
Stirred tank	1.2 liters	direct sparging	hollow fiber	Sf9, IPLB-SF–21 AE	2.0×10^7	1	1.2×10^7	1	IPL–41, Excell 400	Guillaume *et al.*, 1992
Stirred tank	1.2 liters	direct sparging	hollow fiber	SPC-Mb92	5.0×10^7	3	3.0×10^7	1	?	Deramoudt *et al.*, 1994
Airlift	23 liters	direct sparging	?	Sf9	?	0.65	1.0×10^7	1	?	Schlaeger *et al.*, 1992
Airlift	10 liters	direct sparging	flat membrane (Prostak)	IPLB-SF–21 AE	1.0×10^7	3	8.0×10^6	0.44	TC100 +FBS	Hellenbroich, 1995

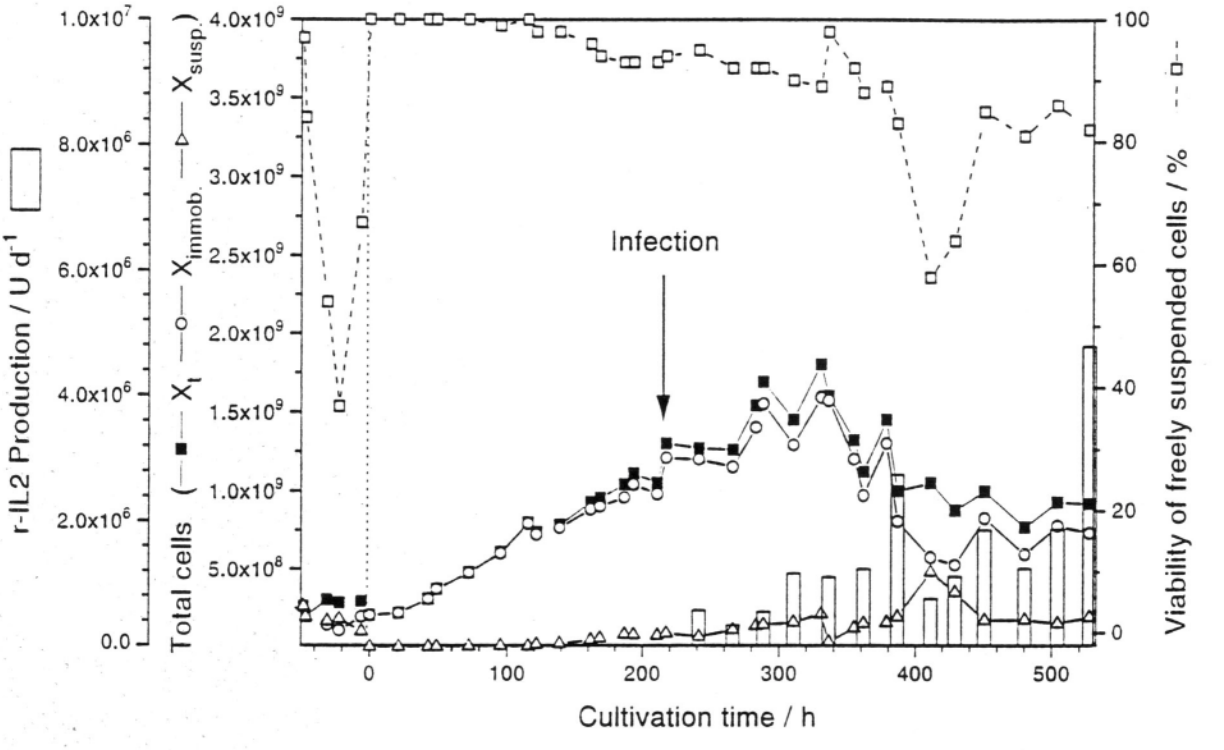

Figure 2. Cultivation of IPLB-SF–21 AE cells in a 0.25-liter fluidized bed reactor for the production of a recombinant interleukin–2 variant (Jäger, unpublished results). Cells were allowed to attach to the carriers (Cytoline–1®) before they were transfered into the bioreactor. Cell growth and product formation are significantly reduced when compared to suspension culture. Similar results were obtained using Siran® carriers instead of Cytoline–1® .

Table 3. Comparison of continuous perfusion culture with batch or fed-batch culture

	Perfusion	Batch/fed-batch
Additional shear forces	appear when using external filtration units	no
Volumetric productivity	medium – high	low – medium
Normalized cell specific productivity	0.5 to 8.5	1
Scale-up potential	limited (internal perfusion) not limited (external perfusion)	not limited
Secreted recombinant proteins:		
Low multiplicity of infection	possible (minimum < 0.001)	possible (infecting at low cell density and resulting in low product yields)
High multiplicity of infection	difficult using low titer virus stocks	recommended
Relative amount of virus required	small	large
Residence time of recombinant proteins (influencing possible protein degradation)	short	long
Non-secreted recombinant proteins:		
Low multiplicity of infection	possible (minimum < 0.1)	possible (infecting at low cell density and resulting in low product yields)
High multiplicity of infection	recommended but difficult using low titer virus stocks	recommended
Relative amount of virus required	average	large
Residence time of recombinant proteins (influencing possible protein degradation)	long (but continuous removal of soluble components of lysed cells)	long

fold) in a 4-liter stirred tank reactor aerated by direct pulse sparging and equipped with a Microgon Cellflo hollow fiber crossflow microfiltration system for perfusion (Cavegn *et al.,* 1992). Densities of approximately 3×10^7 Sf9 cells ml^{-1} could be reached at perfusion rates of up to 5 reactor volumes per day. Usually cells were infected with a MOI of 5 at a density of 1.1×10^7 cells ml^{-1}.

Using a 5-liter BioFlow III stirred tank reactor, aerated by direct sparging and an A/G Technology hollow fiber crossflow system Sf9 cells were grown to a density of 1×10^7 ml^{-1} at relatively low perfusion rates of 0.4 to 0.6 reactor volumes per day (Reuveny *et al.,* 1994). The same crossflow system was also used for high density cultures with medium replacement sometimes combined with fed-batch. The latter cultivation method was suggested to be combined with a second reactor for viral infection in order to obtain maximum protein yields in minimum time.

Using a directly sparged 1.2-liter Cytoflow stirred tank reactor combined with a hollow fiber crossflow microfiltration system Sf9 and IPLB-SF–21 AE cells were grown to maximum densities of 2×10^7 ml^{-1} (Guillaume *et al.,* 1992). Infections with a MOI of 1 were performed at 1.2×10^7 ml^{-1}. The cell specific productivity was comparable to batch cultures. The same bioreactor system has also been used for the cultivation of SPC-Mb 92 cells which were grown to a maximum density of 5×10^7 ml^{-1} (Deramoudt *et al.,* 1994). Infected at a cell density of 3×10^7 ml^{-1} with a MOI of 1 an 8.6 fold increase of the cell specific productivity was observed.

IPLB-SF–21 AE were cultivated in a 10-liter airlift reactor equipped with a Prostak crossflow microfiltration system for medium perfusion (Hellenbroich, 1995). A maximum cell density of 1×10^7 ml^{-1} was reached at a perfusion rate of 3 reactor volumes per day. Cells were infected at a density of 4×10^6 ml^{-1} with a MOI of 0.44. Due to the relatively small virus

inoculum cells continued to grow to 8×10^6 ml^{-1}. The yield of a recombinant interleukin–2 variant was comparable to that obtained in experiments utilizing membrane-aerated stirred tank reactors. In contrast, Sf9 cells grown in a 23-liter airlift reactor with medium perfusion showed a markedly reduced recombinant protein production when compared to low density batch cultures in the same reactor (Schlaeger *et al.,* 1992).

Perfusion processes using external membrane filtration systems are summarized in Table 2. A wide range of culture media with significantly differing nutrient contents has been used for the cultivation of insect cells with medium perfusion. Whereas IPLB-SF-21 AE cells had to be perfused with 3 reactor volumes per day to reach a cell density of 1×10^7 ml^{-1} (Hellenbroich, 1995) the same cell density could be achieved in batch culture by improved media formulations (Ackermann *et al.,* 1994). Due to this fact it is very difficult to compare the different perfusion processes and to extrapolate the potential of the reactor configuration.

In general, external membrane perfusion has proved to be a vital alternative to batch and fed-batch culture. The most critical point appears to be a scale-down of such bioreactor systems. In this case, the circulation frequency of cells becomes too high or the membrane channels become too small and thus generate intolerable shear stress.

Cell immobilization

Different approaches have been used to immobilize insect cells for the production of virus and recombinant proteins. One method is to encapsulate the cells by alginate or other polymers (King *et al.,* 1988, 1989). This procedure is reviewed in more detail in a separate chapter of this issue.

Another method is to grow cells by making use of their potential to adhere to suitable carrier matrices. Cells attached on these carriers can be cultivated in various bioreactor systems depending on the properties of the carriers with respect to size, shape and specific weight. Most frequently used are stationary (fixed, packed) bed bioreactors and fluidized bed bioreactors. Kompier *et al.* (1991a,b) cultivated IPLB-SF–21 AE cells on non-woven fabric discs in a stationary bed reactor for the production of recombinant β-galactosidase. This small reactor consisted of a 50 ml column containing the stationary bed and a 350 ml membrane-aerated medium reservoir. The medium was circulated with a peristaltic pump. Even smaller columns of approximately 20 ml and filled with glass spheres were used for the cultivation of BTI-Tn 5BI–4 cells (Shuler *et al.,* 1990). This column was connected to a small bubble column. The culture medium was circulated by means of a peristaltic pump or by the airlift itself. More recently, this combination of two vessels was replaced by introducing the bed directly into a split flow airlift bioreactor of 210 ml volume (Chung *et al.,* 1993a). Although it is theoretically possible that all of these reactor configurations can be run continuously without complications, all systems were run in batch mode at cell densities of 6×10^6 ml^{-1} or less. All reactor systems contained solid particles without pores as matrices which allow growth of cells only on the surface. However, the question remains unanswered whether insect cells show identical growth and productivity at increased densities especially when limited surface areas force cells to grow in multiple layers.

As an alternative, cells can be immobilized on spherical macroporous carriers made of borosilicate glass (SIRAN® , Schott) or hydrophobic polyethylene (Cytoline–1® , Pharmacia) and cultivated in fluidized bed bioreactors (Jäger, unpublished results). IPLB-SF–21 AE and SPC-Bm36 were cultivated in a 0.25-liter-fluidized-bed reactor described for the cultivation of mammalian cells (Lüllau *et al.,* 1994). Cell densities of approx. 1.4×10^7 ml^{-1} were achieved based on the total reactor volume or 2.5×10^8 ml^{-1} based on the available volume inside of the macroporous carriers. As shown in Figure 2 for the cultivation of IPLB-SF–21 AE cells the growth rate as well as the production rate of the recombinant protein are significantly reduced when compared to perfused suspension culture in stirred tank bioreactors (Jäger *et al.,* 1992). It can be assumed that in contrast to the surrounding culture medium there might be insufficient oxygen concentrations inside the carriers with almost tissue-like cell densities. The unsatisfactory results in the fluidized bed could be a result of the fact that insect cell growth and baculovirus-directed protein production are strongly dependent on sufficient oxygen supply (Deutschmann & Jäger, 1994; Jäger & Kobold, 1995).

Conclusion

High density perfusion culture of insect cells for the production of recombinant proteins has proved to be an attractive alternative to batch and fed-batch processes.

A comparison of the different production processes is summarized in Table 3. Internal membrane perfusion has a limited scale-up potential but appears to the method of choice in smaller lab-scale production systems. External membrane perfusion results in increased shear stress generated by pumping of cells and passing through microfiltration modules at high velocity. However, using optimized perfusion strategies this shear stress can be minimized such that it is tolerated by the cells. In these cases, perfusion culture has proven to be superior to batch production with respect to product yields and cell specific productivity. Although insect cells could be successfully cultivated by immobilization and perfusion in stationary bed bioreactors, this method has not yet been used in continuous processes. In fluidized bed bioreactors with continuous medium exchange cells showed reduced growth and protein production rates.

For the cultivation of insect cells in batch and fed-batch processes numerous efforts have been made to optimize the culture medium in order to allow growth and production at higher cell densities. These improved media could be used in combination with a perfusion process, thus allowing substantially increased cell densities without raising the medium exchange rate. However, sufficient oxygen supply has to be guaranteed during fermentation in order to ensure optimal productivity.

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References

Ackermann M, Hellenbroich DHJ & Jäger V (1994) Improvement of the performance of commercially available insect cell culture media for the Baculovirus-directed production of recombinant proteins in bioreactors. Cytotechnology 14, Suppl. 1: 2.8.

Bédard C, Perret S & Kamen A (1995) Fed-batch culture supports 3×10^7 Sf-9 cells $\times$ mL^{-1}. Presented at the 'Baculovirus and insect cell gene expression conference', Pinehurst, NC, March 26–30, 1995.

Caron AW, Tom RL, Kamen AA & Massie B (1994) Baculovirus expression system scaleup by perfusion of high-density Sf–9 cell cultures. Biotechnol. Bioeng. 43: 881–891.

Cavegn C, Blasey HD, Payton MA, Allet B, Li J & Bernard AR (1992) Expression of recombinant proteins in high density insect cell cultures. In: Spier RE, Griffiths JB & MacDonald C (eds) Animal cell technology: Developments, processes and products (pp. 569–578) Butterworth-Heinemann, Oxford.

Cavegn C & Bernard AR (1992) A perfusion process for high density insect cell cultures. In: Vlak JM, Schlaeger E-J & Bernard AR (eds) Baculovirus and recombinant protein production processes (pp. 262–273) Editiones Roche, Basel.

Chung IS, Taticek RA & Shuler ML (1993) Production of human alkaline phosphatase, a secreted, glycosylated protein, from a Baculovirus expression system and the attachment-dependent cell line *Trichoplusia ni* BTI-Tn 5B1–4 using a split-flow, air-lift bioreactor. Biotechnol. Prog. 9: 675–678.

Chung IS & Shuler ML (1993) Effect of *Trichoplusia ni* BTI-Tn 5B1–4 cell density on human secreted alkaline phosphatase production. Biotechnol. Lett. 15: 1007–1012.

de la Broise D, Noiseux M, Lemieux R & Massie B (1991) Long-term perfusion culture of hybridoma: A 'grow or die' cell cycle system. Biotechnol. Bioeng. 38: 781–787.

Deramoudt F-X, Monnet S, Rabaud J-N, Quiot J-M, Cerutti M, Devauchelle G & Kaczorek M (1994) Production of a recombinant protein in a high density insect cell Cytoflow reactor. In: Spier RE, Griffiths JB & Berthold W (eds) Animal cell technology: Products of today, prospects for tomorrow (pp. 222–226) Butterworth-Heinemann, Oxford.

Deutschmann S & Jäger V (1991) High density suspension culture of insect cells in a stirred bioreactor. In: Sasaki R & Ikura K (eds) Animal cell culture and production of biologicals (pp. 151–158) Kluwer, Dordrecht.

Deutschmann SM & Jäger V (1994) Optimization of the growth conditions of Sf21 insect cells for high-density perfusion culture in stirred-tank bioreactors. Enzyme Microb. Technol. 16: 506–512.

Fraune E, Fenge C, Kuhlmann W & Broly H (1991) Development of perfusion bioreactors for high density cultures. In: White MD, Reuveny S & Shafferman A (eds) Biologicals from recombinant microorganisms and animal cells (pp. 159–164) VCH, Weinheim.

Guillaume JM, Couteault N, Hurwitz DR & Crespo A (1992) High density insect cell homogenous perfusion culture for recombinant proteins production. In: Vlak JM, Schlaeger E-J & Bernard AR (eds) Baculovirus and recombinant protein production processes (pp. 285–296) Editiones Roche, Basel.

Hellenbroich DHJ (1995) Einsatz von Airlift-Reaktoren für Produktionsverfahren mit tierischen Zellkulturen. Ph.D. Thesis, Brunswick Technical University.

Jäger V, Grabenhorst E, Kobold A, Deutschmann SM & Conradt HS (1992) High density perfusion culture of insect cells for the production of recombinant glycoproteins. In: Vlak JM, Schlaeger E-J & Bernard AR (eds) Baculovirus and recombinant protein production processes (pp. 274–284) Editiones Roche, Basel.

Jäger V & Kobold A (1995) Propagation of *Spodoptera frugiperda* cells (Sf9) and production of recombinant proteins with the Baculovirus expression system using improved spinner flasks with membrane aeration. Biotechnol. Techniques 9: 435–440.

King GA, Daugulis AJ, Faulkner P, Bayly D & Goosen MFA (1988) Growth of Baculovirus-infected insect cells in microcapsules to a high cell and virus density. Biotechnol. Lett. 10: 683–688.

King GA, Daugulis AJ, Goosen MFA, Faulkner P & Bayly D (1989) Alginate Concentration: A key factor in growth of temperature-sensitive Baculovirus-infected insect cells in microcapsules. Biotechnol. Bioeng. 34: 1085–1091.

Klöppinger M, Fertig G, Fraune E & Miltenburger HG (1990) Multistage production *of Autographa californica* Nuclear Polyhedrosis Virus in insect cultures. Cytotechnology 4: 271–278.

Klöppinger M, Fertig G, Fraune E & Miltenburger HG (1991) High density perfusion culture of insect cells for production of Baculovirus and recombinant protein. In: Spier RE, Griffiths JB &

Meignier B (eds) Production of biologicals from animal cells in culture (pp. 470–476) Butterworth-Heinemann, Oxford.

Kompier R, Tramper J & Vlak JM (1988) A continuous process for the production of Baculovirus using insect-cell cultures. Biotechnol. Lett. 10: 849–854.

Kompier R, Kislev N, Segal I & Kadouri A (199la) Use of a stationary bed reactor and serum-free medium for the production of recombinant proteins in insect cells. Enzyme Microb. Technol. 13: 822–827.

Kompier R, Kislev N, Segal I & Kadouri A (1991b) The use of a non-woven fabric carrier for the production of β-galactosidase from insect cells. In: White MD, Reuveny S & Shafferman A (eds) Biologicals from recombinant microorganisms and animal cells (pp. 271–274) VCH, Weinheim.

Lüllau E, Biselli M & Wandrey C (1994) Growth and metabolism of CHO-cells in porous glass carriers. In: Spier RE, Griffiths JB & Berthold W (eds) Animal cell technology: Products of today, prospects for tomorrow (pp. 252–255) Butterworth-Heinemann, Oxford.

Maiorella B, Dorin G, Carion A & Harano D (1991) Crossflow microfiltration of animal cells. Biotechnol. Bioeng. 37: 121–126.

Massie B, Tom R & Caron AW (1992) Scale-up of a Baculovirus expression system: Production of recombinant protein in perfused, high-density Sf9 cell cultures. In: Vlak JM, Schlaeger E-J & Bernard AR (eds) Baculovirus and recombinant protein production processes (p. 234) Editiones Roche, Basel.

Reuveny S, Kemp CW & Shiloach (1994) High cell density in insect culture. In: Spier RE, Griffiths JB & Berthold W (eds) Animal cell technology: Products of today, prospects for tomorrow (pp. 494–503) Butterworth-Heinemann, Oxford.

Schlaeger E-J, Loetscher H & Gentz R (1992) Production of recombinant soluble human TNF receptor using the Baculovirus-insect cell expression system. In: Spier RE, Griffiths JB & MacDonald C (eds) Animal cell technology: Developments, processes & products (pp. 562–568) Butterworth-Heinemann, Oxford.

Schütz C, Jäger V, Driesel AJ & Wagner R (1991) Cultivation of insect cell lines in stirred membrane reactors. In: Spier RE, Griffiths JB & Meignier B (eds) Production of biologicals from animal cells in culture (pp. 460–466) Butterworth-Heinemann, Oxford.

Shuler ML, Cho T, Wickham T, Ogonah O, Kool M, Hammer DA, Granados RR & Wood HA (1990) Bioreactor development for production of viral pesticides or heterologous proteins in insect cell cultures. Ann. N.Y. Acad. Sci. 589: 399–422.

Tramper J, van den End EJ, de Gooijer CD, Kompier R, van Lier FLJ, Usmany M & Vlak JM (1990) Production of baculovirus in a continuous insect-cell culture. Bioreactor design, operation, and modeling. Ann. N.Y. Acad. Sci. 589: 423–430.

van Lier FLJ, van den End EJ, de Gooijer CD, Vlak JM & Tramper J (1990) Continuous production of Baculovirus in a cascade of insect-cell reactors. Appl. Microbiol. Biotechnol. 33: 43–47.

van Lier *FLJ,* van der Meijs WCJ, Grobben NG, Olie RA, Vlak JM & Tramper J (1992) Continuous β-galactosidase production with a recombinant Baculovirus insect-cell system in bioreactors. J. Biotechnol. 22: 291–298.

van Lier FLJ, van Duijnhoven GCF, de Vaan MMJACM, Vlak JM & Tramper J (1994) Continuous β-galactosidase production in insect cells with a p10 gene based Baculovirus vector in a two-stage bioreactor system. Biotechnol. Prog. 10: 60–64.

Wood HA, Johnston LB & Burand JP (1982) Inhibition of *Autographa californica* Nuclear Polyhedrosis Virus replication in high-density *Trichoplusia ni* cell cultures. Virology 119: 245–254.

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Immobilization of insect cells

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Introduction

The production of baculoviruses and recombinant proteins in insect cell culture has been mostly carried out in culture systems with freely-suspended cells. Suspended culture systems usually suffer from a number of disadvantages, such as the fluid mechanical cell damage from agitation and air-sparging, low cell and product concentrations, and low productivity. Another limitation is that the method is only suitable for the growth of non anchorage-dependent cell (e.g., Sf–9 cells). These drawbacks can be eliminated with the exploitation of cell immobilization techniques, such as the attachment of cells on solid surfaces, entrapment of cells in polymer gels, and encapsulation of cells in artificial membranes. With immobilized cells, a 10-fold or more increase in the cell and production concentrations can be achieved (e.g., Dean *et al*., 1987; Reiter *et al*., 1991; and Park & Stephanopoulos, 1992). The technology will also allow for simpler cell-medium separation and product purification processes. In addition, immobilization of cells in porous supports and microencapsules could also prevent the cell from mechanical damage due to mechanical agitation and gas sparging.

There have been extensive studies on immobilization of animal cells and its application in large-scale production of mammalian cells and associated products, such as hybridoma cells in the production of monoclonal antibodies (Reuveny, 1985; Heath and Belfort, 1987). The development of immobilized insect cell culture, however, is still in its infant stage. This is not surprising since it is only recently that insect cell culture has been recognized as an important tool in the biotechnology industry (Luckow and Summers, 1988). Other factors responsible for the fewer studies on insect cell immobilization may be related to the type of insect cells used for large-scale production and the mechanism of virus and recombinant protein production. The cell lines used predominantly in baculovirus and recombinant protein production, i.e., those derived from *Spodoptera frugiperda* insect, Sf–21 and Sf–9, grow well in suspension, which undermines the advantages of immobilized culture. On the other hand, production of cell products involves viral infection and subsequent lysis of the cell. Immobilized cell culture can thus only be used for one batch operation. With the development of anchorage-dependent insect cells for virus and protein production, and processes of producing secreted cell products without cell lysis, immobilization technology will become more attractive for large-scale production.

In this chapter, we will primarily focus on techniques for animal cell immobilization in particulate supports, including microcarrier beads and microcapsules, with emphasis on their applications in insect cell culture. Typical immobilization materials and methods, immobilized cell bioreactors and the major concerns associated with each technique will be addressed. The review will conclude with an outline of the studies conducted in our laboratory on insect cell immobilization techniques.

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Cell immobilization systems

A variety of systems can be employed for cell and enzyme immobilization. These include, for example, microcarriers (van Wezel, 1967), gel entrapment (Guo *et al.*, 1990), hollow fibers (Knazek *et al.*, 1972), and encapsulation (Lim and Moss, 1981). In principle, these methods fall into one of two categories: entrapment in a three dimensional polymer matrix or capture behind a semipermeable membrane. Among these methods, microcarriers and gel entrapment are probably the most popular. However, one common problem with both is the leakage of the immobilized biocatalyst. Hollow fibers and microencapsulation overcome this problem by separating the cells from converted medium with a semipermeable membrane. Our attention in this section will be on the immobilization of animal cells in particulate carriers, including microcarriers, porous beads and microcapsules (Table 1).

Microcarriers

The introduction of microcarriers to animal cell culture is a major breakthrough in the development of anchorage-dependent animal and mammalian cell culture processes. The traditional method for large-scale production of anchorage-dependent animal cells was to grow the cells on the inside surfaces of rotating bottles, or roller bottles. Major disadvantages of the roller bottle system include a very low surface-to-volume ratio, variable quality of the cell products and extensive labor costs required for processing large numbers of bottles: In a microcarrier culture, on the other hand, cells are attached on the surface of small carrier beads suspended in the medium. The small carrier beads, with a diameter of a few hundred microns, provide a large surface area for cell attachment. For instance, the surface-to-volume ratio of a microcarrier culture is about $30\ cm^{-1}$ at 5 g/l particle concentration, compared to 1.2 cm^{-1} for a roller bottle (Butler, 1987). Apart from a significant increase in the surface-to-volume ratio, use of microcarriers has made it possible to cultivate anchorage-dependent cells in a homogeneously suspended condition, which greatly facilitates large-scale production.

Microcarrier culture technology was first reported by van Wezel (1967), nearly three decades ago. He cultivated mammalian cells adhering on DEAE-Sephadex® A–50 beads in suspension culture. The microcarrier chosen by van Wezel (1967), i.e., DEAE-Sephadex A–50, was designed primarily for ion exchange chromatography. These microcarrier beads consist of a dextran matrix with a net positive surface charge which is considered necessary for cell attachment. The microcarriers were found suitable for cell growth at particle concentrations no greater than 1 g/l. Exceeding that level of beads loading was found to cause inhibitory effects on cell growth. This adverse effect was believed to be related to the charge density on the bead surface. By reducing the surface charge of the microcarriers, Levine *et al.* (1977) were able to eliminate this toxic effect, achieving cell attachment and growth at particle concentrations up to 5 g/l. This breakthrough made it feasible to grow anchorage-dependent cells to high cell densities to suspension culture bioreactors.

Polymeric products, such as dextran (polysaccharide), cellulose, Gelatin (polysaccharide) and plastic (polystyrene) have been primary matrix materials for cell immobilization (Table 2). Particularly, the dextran-based microcarriers, Cytodex 1, 2, 3 (Pharmacia Fine Chemicals, Uppsala, Sweden), have been most widely used in large-scale cell culture as well as laboratory research. The most important physical and chemical properties of microcarriers particles may include surface charge, density and size. Surface charge, as mentioned earlier, is crucial for cell attachment and cell growth on the microcarriers. The optimal charge density is 2.0 meg/g (Butler, 1987). Usually the density of the microcarrier beads is relatively low (typically 1.03 g/l, slightly higher than the liquid medium) to facilitate suspension with gentle agitation. Heavier materials requiring high stirring speeds to suspend may cause cell damage. The diameter of the microcarriers should conform to a high surface-to-volume ratio, there should be sufficient surface area on each microcarrier for a single cell to divide for several generations, the manufacture process should also be inexpensive. Diameters in the range of 150–220 have been found to be desirable. Other properties, such as microcarrier transparency, size distribution and rigidity are also important concerns for cell attachment, growth in suspension, visual observation, and cell growth and viability measurement. In addition, non-toxicity is essential for cell survival as well as for medical applications of cell products.

A major drawback with current microcarriers is that they cannot be reused (i.e., cleaning and sterilization). This results in considerable increase in the cost of the culture process (e.g., \$50/l with 5 g/l beads loading, Glacken *et al.*, 1983). In addition, one of the major concerns with microcarrier culture is mechanical dam-

Table 1. Immobilization techniques for animal cell culture

Method	Cell type	Culture system	Concerns
Microcarriers	Anchorage-dependent	Stirred suspension	Shear damage
Porous beads	Anchorage-dependent	Stirred suspension Fixed/fluidized bed	Oxygen supply efficiency
Micro-encapsulation	Anchorage-independent	Stirred suspension	Nutrient/oxygen transfer through membrane Encapsulation complexity

Table 2. Properties of typical animal cell immobilization systems

Materials	Size (mm)	Density (g/mL)	Application	References
Microcarriers	0.10	1.03-	All kinds anchorage-	van Wezel (1967)
DEAE-Dextran	0.23	1.10	dependent mammalian	Pharmacia (1981)
DEAE-Cellulose			cells, e.g., hybridoma,	Butler (1987)
Geltin			CHO, VERO, BHK,	Agathos *et al.* (1989)
Collagen			and insect: Tn–5B1–4	Wickham and Nemerow (1993
Polystyrene			(T.ni), C7–10	
Polyacrylamide			(*Aedes albopictus*)	
Porous Carriers				
Ceramic	0.59	1.50	AtT20 (rat pituitary)	Park & Stephanopoulus (1993)
(magnesium alminate)	0.85			
Glass	1.00–2.00		Hybridoma	Reiter *et al.* (1992)
Polystyrene	0.50–1.00		VERO, hybridoma	Lee *et al.* (1992)
Polyethylene	1.20–1.50	1.15	CHO	Reiter *et al.* (1991)
Collagen	0.50	1.60	Hybridoma, CHO	Dean *et al.* (1987)
(entrapped with heavy metal)				
Microcapsules	0.70–1.00/		Pancreatic islets	Lim and Moss (1981)
Alginate-polylysine	controllable		Hybridoma	Posillico (1986)
			Hybridoma	Goosen *et al.* (1989)
			Insect Sf–21	King *et al.* (1989)

age of the cell attached on the carrier surface due to fluid shear stresses and possibly collision between the carrier beads (Croughan *et al.*, 1988).

The kinetics of animal cell growth on microcarriers was found to be essentially the same as in monolayer culture, but much higher cell densities can be achieved if the medium is replenished (e.g., medium perfusion) (van Wezel, 1972). The choice of a suitable microcarrier depends on the type of cells (e.g., plating efficiency and morphology) and the purpose of the culture (product harvest or cell biology studies). This will in turn affect the choice of the culture vessels. Although in principle microcarrier culture can be contained in virtually any type of cell culture vessel (static monolayer or suspension), its advantages are best realized in stirred suspension culture. To avoid cell detachment and fluid mechanical damage, the stirring action should be gentle, and evenly distributed, with minimum agitation.

Porous carriers

Although microcarrier technology has many advantages for animal cell culture, it still has some drawbacks. First of all, the technique is only suitable for anchorage-dependent cells. Secondly, cells attached

on the surface of the microcarriers are susceptible to detachment and shear damage in stirred condition. Recently, there have been many reports on the use of porous carrier supports, made of various materials (e.g., ceramic, glass, polystyrene and collagen) for the immobilization of anchorage-dependent and non anchorage-dependent animal cells (Table 2). Although these porous carriers have been frequently put into the same category as microcarriers, a separate discussion is presented here in terms of the differences between the two in physical properties and size ranges, apart from the porosity.

As mentioned earlier, most microcarriers consist of polymeric matrixes, with densities slightly higher than the aqueous media, and diameters in the range of 100–230 μm. Many porous carriers, however, are made of inorganic materials, such as ceramic and glass. Their sizes tend to be much larger than microcarriers (>0.5 mm in diameter). Since inorganic carriers are usually much heavier and more rigid than polymeric microcarriers, they can be used in fixed or packed bed and fluidized bed reactors. The primary advantages of the porous carriers are their high void volume and large surface area.

Various porous carriers have been developed and commercialized. Verax, Cultispher, and Siran microsphere are typical examples. The Verax Corporation (Dean *et al.*, 1990) developed a porous microsphere made from natural collagen. It was sponge-like and approximately 500 μm diameter. The sponge-like collagen matrix, was presumed to mimic the *in vivo* extracellular matrix of mammals. In order to use the microspheres in a fluidized bed, they entrapped heavy metal particles in the beads, increasing the specific gravity to 1.6. Work still needs to be done, and on the effect of chemical modification of the matrix surface on productivity.

Microcapsules

Microencapsulation of cells is the entrapment of the cells in semipermeable membrane capsules. This capsule membrane, typically of alginate and poly-L-lysine (PLL), selectively allows small molecules such as oxygen and nutrients to diffuse freely to the cells, preventing the passage of large molecules and cells. Both the size of the microcapsules and the porosity of the capsule membrane may be varied to accommodate various reactant-product systems. Microencapsulation of living cells and tissues was first reported by Chang (1963) and more recently by Lim and Moss (1981), who used alginate-polylysine microencapsulated pancreatic islets injected into diabetic pancreas of animals. Later development and application has been mainly evolved through modifications of this procedure (ref).

The microencapsulation of animal cells with the alginate-polylysine membrane usually involves two main steps (Lim and Moss) (Figure 1). The first step is to immobilize (entrap) the cells in alginate beads, and the second is to coat the gel beads with a biopolymer (polylysine) membrane. To prepare the alginate beads, the animal cells are suspended in an alginate (e.g., Na alginate) solution. King *et al.* (1987) showed that the membrane molecular weight cutoff of alginate-polylysine (PLL) microcapsules could be controlled from 60×10^3 to about 300×10^3 by varying the molecular weight of the PLL, the alginate-PLL reaction time, and the PLL concentration. These findings led to the development of a multiple-membrane microcapsule system which had a reduced intracapsular alginate concentration (King *et al.*, 1989). Compared to the conventional single-membrane system, higher intracapsular cell densities and product concentrations (about 300%) were obtained in approximately one half the cell culture period. In addition there appeared to be better retention of the cell product by the multiple membrane microcapsule. Posillico (1986) employed the alginate-PLL microcapsules in large-scale production of monoclonal antibodies from hybridoma cells. He indicated, however, that the cells appeared to grow preferably near the interior surface of the capsule membrane. He suspected that this may have been due to mass transfer limitations during the cell culture or to the presence of viscous intracapsular alginate solution.

While most of the animal cell microencapsulation work had been done with mammalian cells, Goosen's group (King *et al.*, 1988; Goosen, 1993) was the first to apply the technology to insect cell culture. There is, however, a major technical barrier to the use of the encapsulated cells for the production of baculoviruses and recombinant proteins. Since the viruses cannot pass through the capsule membrane, the cells must be infected prior to microencapsulation, which would immediately shut off any further cell growth. In Goosen's work, the difficulty was circumvented by using a temperature-sensitive baculovirus, which was not infectious except at a certain temperature (e.g., 27 °C). Therefore, by maintaining a slightly higher temperature (33 °C) in the first few days, they were able to grow the encapsulated cells (exposed to the viruses prior to entrapment) to a higher concentration, and then initiated the infection by shifting the temper-

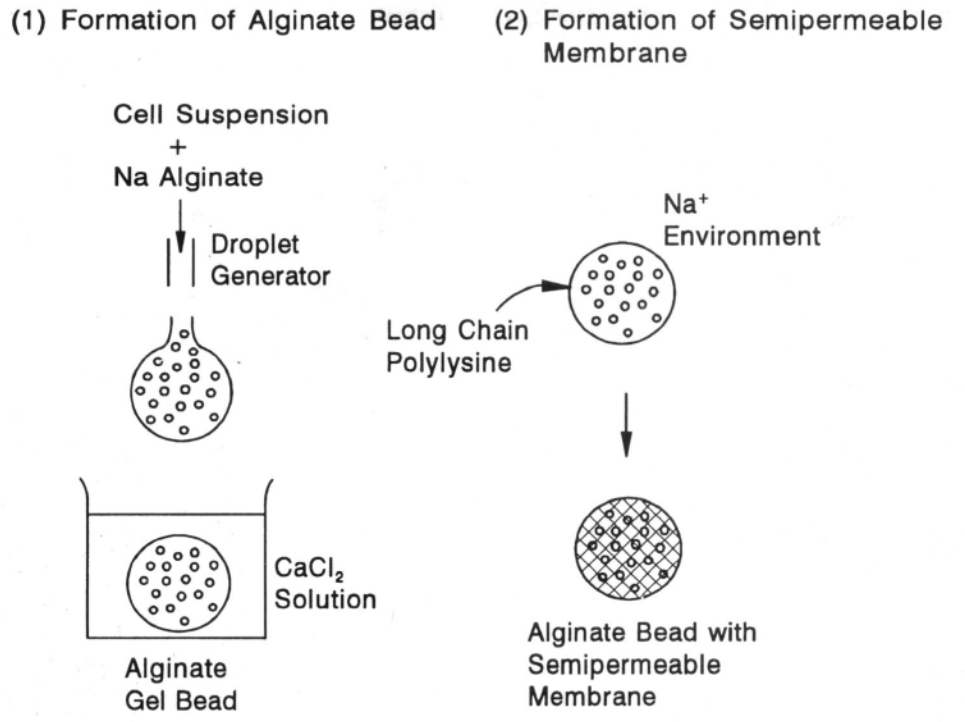

Figure 1. Procedure for microencapsulation of animal cells with alginate polylysine polymers (Posillico, 1986).

ature to 27 °C. The cell concentrations in the capsules reached up to 8×10^7 cells/ml capsules, along with virus titres of 10^9 plaque-forming units/ml capsules.

Immobilized insect cell culture systems

The insect cell lines that have been used predominantly in biochemical research and production of baculoviruses and recombinant proteins are those derived from insects *Spodoptera frugiperda* (Sf–21, Sf–9) and *Trichoplusla ni* (TN–386). These cells are not anchorage-dependent, and can grow in both surface–attached monolayer and suspension cultures. Successful large-scale culture has been achieved in anchorage-dependent roller bottlers (Vaughn, 1976; Weiss *et al.*, 1981) as well as stirred/sparged suspension bioreactors (Wu *et al.*, 1992). With surface-attached culture, the attachment forces between the insect cells and the solid surface are usually weaker than for mammalian cells (Agathos *et al.*, 1989). To detach the cells from the solid support, therefore, only gentle mechanical force is required while the enzyme trypsin has to be used for detachment of anchorage-dependent mammalian cells from their substratum. Hence, these insect cell lines may not be readily grown in suspended microcarrier culture since the cells are liable to detach from the carrier surface under agitation.

A few insect cell lines other than the non anchorage-dependent Sf and T.ni cells have been immobilized in microcarriers and other particulate supports. Agathos *et al.* (1989) immobilized insect cells *Aedes albopictus* (mosquitos) on collagen-based microsphere, i.e., Verax beads (Dean *et al.*, 1990) and cultivated the cells in suspension (spinner reactor). The growth rate and the maximum cell density of the immobilized cells were similar to those of freely suspended cells. Shuler *et al.* (1989) grew T.ni insect cells (TN–5B1–4) in a packed bed-air-lift bioreactor, with the cells attached on 3-mm glass beads. The reactor system consisted of a packed bed connected to a bubble column. Medium circulation in the reactor system was realized with air sparging in the bubble column. Since the cells were not directly exposed to air-sparging, the cell damage problem was avoided while effective gas exchange in the culture medium could be achieved. There was no cell detachment observed 70 hour post-infection of anchorage-dependent insect cells and recombinant proteins. How-

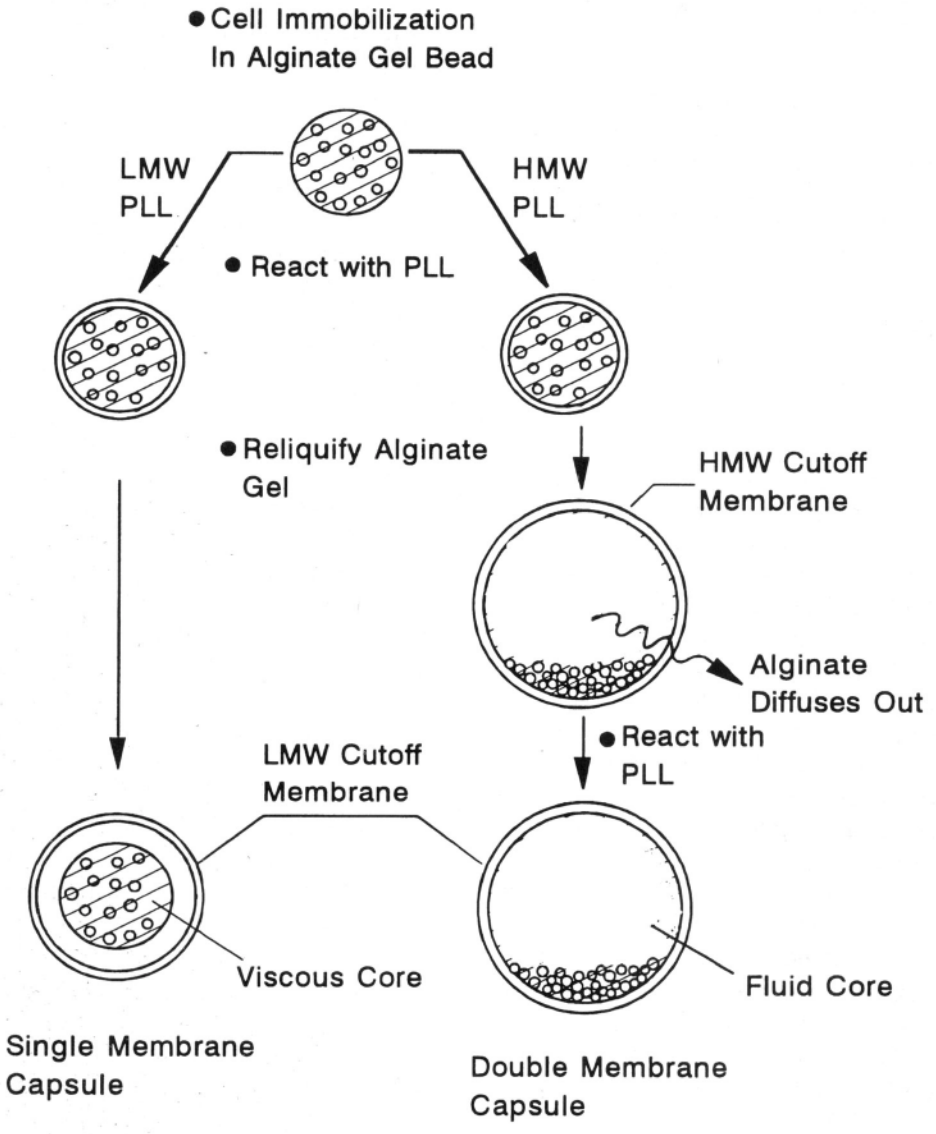

Figure 2. Encapsulation of insect cells in single- and multiple-membrane microcapsules (King *et al.*, 1989).

ever, there was not sufficient data to show if this culture system could maintain cell growth, virus and recombinant protein production comparable to other culture systems.

Recently Wickham and Nemerow (1993) cultivated anchorage-dependent insect cells (TN–5B1–4) in microcarrier-coated roller bottles and in suspended microcarriers in spinner-flasks. They evaluated two types of microcarriers, DEAE-based Dormacell (JRH Biosciences, Lenexa, KS) and collagen-coated Cytodex (Pharmacia). In roller bottles, the collagen-coated microcarriers did not adhere to the roller bottle surface. The cells also attached quickly and grew well on the DEAE-based carriers. The cells preferentially grew between the carrier beads, utilizing only a fraction of the available bead surface area. In suspension culture, they observed good cell attachment on the DEAE-based and collagen-coated microcarriers. However, with DEAE-based carriers, large aggregates of microcarriers formed shortly (within 1 hour) after cell attachment. This was followed by cell lysis.

Development of an insect cell encapsulation process

In this section, an outline is given of research effort, in our laboratory, to develop microencapsulation techniques suitable for insect cell culture and baculovirus production (King *et al*., 1989; Goosen *et al*., 1993). Topics which will be covered include the selection and biocompatibility of the polymer, immobilization, diffusion of nutrients through the capsules, and the growth of cells and viruses in the capsules.

Encapsulation of insect cells

Two types of microcapsules were developed to immobilized insect cells, one was the single-membrane and the other was multiple-membrane capsule. The encapsulation procedure for the *single-membrane microcapsules* was a modification of the method originally developed by Lim and Moss (1981), described earlier in this chapter. Insect cells (IPLB-Sf–21) grown to subconfluent in 75-cm^2 tissue culture flasks were resuspended in fresh medium (TC–100), and spun down at 1000 rpm for 10 min. The pellet was resuspended in 3 mL of 1.4% sodium alginate solution. (All the percentage concentration values presented in this section represent the weight-to-volume ratios, unless otherwise specified). This viscous solution was then extruded into 1.5% $CaCl_2$ solution through a 22-gauge needle to a coaxial air-jets/syringe pump droplet generator. Droplets forming at the needle tip were pulled off by a stream of air rapidly flowing through the annular region between the two coaxial cylinders. The droplets hardened into calcium alginate beads in the calcium chloride solution. With the beads settled from suspension, the supernatant was aspired off.

Following rinses in 0.1% CHES and 1.1% $CaCl_2$ and KC1 solution, the gel beads obtained as above were reacted with 0.05% PLL solution ($M_v = 2.2 \times 10^4$). After this, beads were rinsed with KC1 solution. Reaction with 0.03% sodium alginate formed an outer layer on the membrane. The beads were then washed twice with KC1 solution, followed by incubation in 0.05 M sodium citrate to reliquify the interior of the capsules. The capsules were washed with KC1 solution to remove the excess citrate and then incubated in equal volumes of KC1 solution and 2xTC100 medium to allow residual of the intracapsular alginate to diffuse out of the capsules and to allow the capsules to expand toward their equilibrium state. The capsules were then added to complete TC100 medium and incubated at 33 °C.

Preparation of the *multiple-membrane microcapsules* started with the same procedure as the single-membrane capsules, except that a higher molecular weight PLL ($M_v = 2.7 \times 10^5$) was used in the membrane-forming step. After the citrate step, however, the capsules were incubated in an equal volume of KC1 solution and 2xTC100 medium and rocked end to end for 20–30 minutes, allowing the lower molecular weight alginate to diffuse out. The capsules also swelled toward their equilibrium state. Following rinses with KC1 solution, the membrane molecular weight cutoff was reduced by reacting the microcapsules with 0.02–0.15% PLL of $M_v = 2.2 \times 10^4$. The microcapsules were finally rinsed with 0.03% sodium alginate solution. The capsules were then washed with KC1 solution to remove unbound alginate, added to complete TC100 medium and incubated at 33 °C.

Growth of insect cells and viruses in microcapsules

Prior to encapsulation, the cells were incubated with a temperature-sensitive baculovirus, ts10, (multiplicity of infection, MOI, of 0.05–0.10) at 33 °C to allow absorption of the viruses to the cells. This infected cell suspension was encapsulated as previously described and cultured at 33 °C, a non-permissive temperature of ts10 virus replication. Five days later, the encapsulated cells were transferred to a 27 °C incubator to initiate ts10 virus replication. Insect cell density was determined daily by removing about 50 capsules from the growth medium using a Pasteur pipette. The average capsule diameter was measured for determination of the intracapsular volume. Excessive liquid surrounding the capsules was then removed, and the capsules were ruptured, then trypan blue dye was added. The cell density in this solution was determined using a hemocytometer.

The single- and multiple-membrane capsules produced with an initial alginate concentration of 1.5% were spherical in shape and showed no surface irregularities. The cells in the single-membrane capsules remained dispersed throughout the volume of the capsule and, after 2 days in culture, became enlarged and dark in color. Recovery of some of cells from these capsules and subsequent staining with trypan blue indicated that few cells were living. In contrast, the cells in the multiple-membrane capsules tended to settle to the bottom of the capsule, indicating that the intracapsular viscosity was lower. After 2 days in culture, these cells appeared to be healthy (supported by exclusion of trypan blue dye), though little sign of growth could

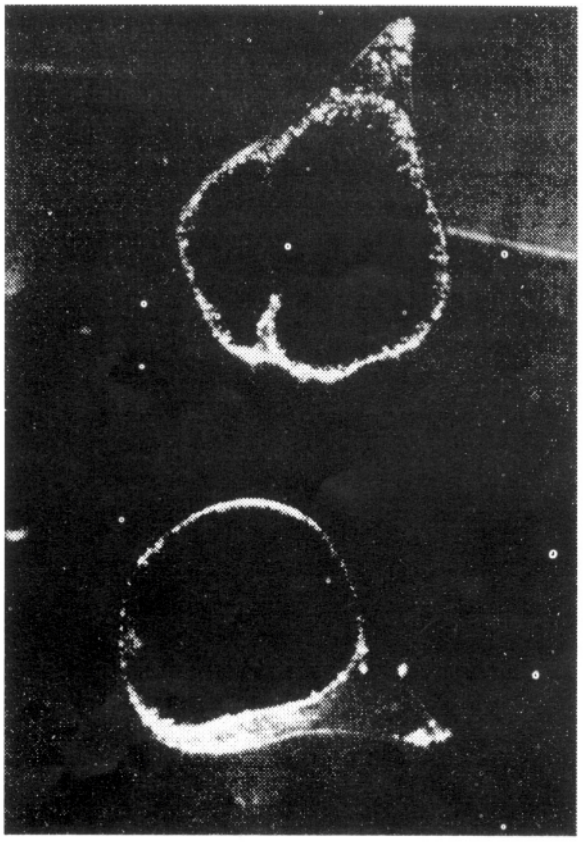

Figure 3. Growth of insect cells in the multiple-membrane microcapsules at day 10. Initial sodium alginate concentration 0.7% (King *et al.*, 1989).

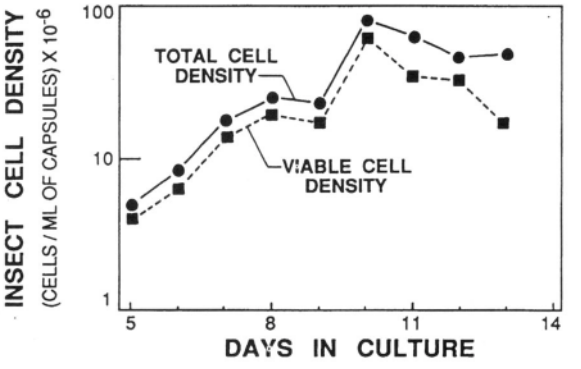

Figure 4. Intracapsular insect cell density as a function of time. Batch culture consisted of 1-ml capsules in 30 ml medium (King *et al.*, 1989).

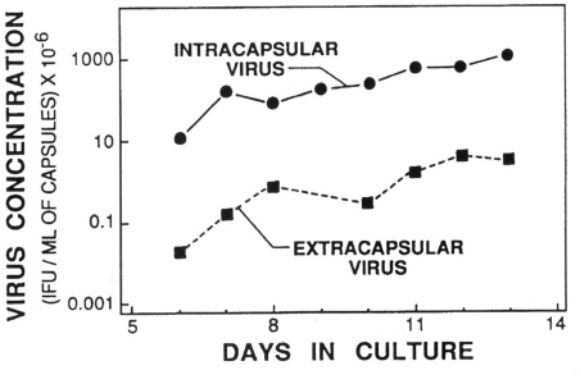

Figure 5. Intracapsular and extracapsular virus concentration as a function of culture time. At day 5, the culture temperature was dropped from 33 to 27 °C to initiate virus production in the infected cells (King *et al.*, 1989)

be observed. The doubling time of the insect cells normally is between 17 and 24 hours.

The capsules produced using a 0.7% alginate-TC100-cell mixture were non-spherical and tended to have pointed tail, and creased sides. This non-sphericity, due to the low alginate concentration and hence low viscosity, was most prevalent when the alginate concentration was 0.5%. The capsules produced with a single, high molecular weight cutoff membrane (and either 0.7 or 1.4% alginate) tended to have week membranes which broke easily and, consequently, allowed the cells to escape and proliferate in the medium outside the capsules. In the few capsules that did not rupture, the infected cells grew in isolated clumps on the inside surface of the membrane.

The multiple-membrane capsules (produced at an initial alginate concentration of 0.7%) were much stronger and more flexible than their single membrane counterparts, as judged by pinching the capsules with fine-tipped forceps. Consequently, there were fewer ruptured capsules, and significantly reduced number of cells found in the supernatant. Cells grew and virtually filled the entire capsule (Figure 3), reaching densities of 8×10^7 cells/ml (Figure 4). In comparison, maximum cell densities in suspension culture were at least ten times lower. The specific growth rate of encapsulated cells was $0.55\ d^{-1}$, and was only slightly lower than that of suspended cells in shaker-flasks ($0.66\ d^{-1}$). The $TCID_{50}$ assay revealed that the virus titer in the capsules reached approximately 1×10^9 infectious units (PFU)/ml (about 20 PFU per cell). The titer of the supernatant was lower by a factor of 300 (Figure 5). This indicates that virtually all of the viruses (more than 99%) were retained within the capsules.

Therefore, much better cell and virus growth was obtained with the multiple-membrane capsules and at a lower alginate concentration. The improved cell and

Table 3. Biocompatibility tests on PLL and encapsulation solutions (King *et al.*, 1989)

Solution	Percentage of cells living (n = 3)
PLL Solution (0.05%, w/v)	
$M_v = 2.2 \times 10^4$	100
$M_v = 2.7 \times 10^5$	24±1
Citrate	100
CHES	100
KCl	100

Table 4. Biocompatibility tests, insect cells suspended in calcium alginate (King *et al.*, 1989)

Alginate concentration (w/v) %	Cell Viability
1.50	All cells enlarged and dark; most dead
1.30	All cells enlarged and dark; most dead
1.00	Some cell dark and granular while some are round and healthy; some cells have multiplied to form small masses
0.75	Good cell growth; many cell masses
0.50	Good cell growth; many cell masses

virus growth was presumably due to the lower intracapsular alginate content, which would increase nutrient and oxygen transfer within the capsules. On the other hand, alginate concentrations of 1.0% or higher have been found to be toxic to the insect cells or inhibitory to cell growth. This will be further discussed in the following section on biocompatibility tests of various chemicals involved in the encapsulation processes.

Biocompatibility tests and mass transfer limitations

Biocompatibility tests were conducted on the encapsulation solutions and the polymers by measuring their effects on cell growth and viability. The KC1, CHES, $CaCl_2$ and citrate solutions at specified concentrations had no apparent effect on cell growth or viability. While exposure of the cells to PLL of M_v 2.2×10^4 resulted in virtually no loss of cell viability, exposure to PLL of $M_v = 2.7 \times 10^5$ resulted in a 76% cell viability loss (Table 3). Cells exposed to alginate showed a decrease in viability, with the extent depending on the alginate concentration. On mixing the 2xTC100 medium and the alginate solutions, a gel-like material was formed, due presumably to the interaction between the calcium ions present in the 2xTC100 medium (2.64 g/L) and the alginate. The gel tended to be more solid as the alginate concentration was increased from 0.5 to 1.5%. Cells suspended in the mixtures of alginate and TC100 at final alginate concentrations of 1.0, 1.3 and 1.5% showed little or no growth, and usually appeared dark and granular after 2–3 days (Table 4). Trypan blue staining indicated that few of the cells were alive. Cell growth, however, was observed at lower alginate concentrations, 0.5 and 0.75%.

There are two possible explanations for the effects of alginate concentration on insect cells. One is that the polymer (Na alginate) is toxic to the cells. However, the tests of Smith *et al.* (1989) showed that viability of Sf–21 insect cells was not insignificantly influenced by the polymer at a concentration of either 0.7 or 1.4%, though the maximum cell-polymer contact time was only 20 min. The other explanation is that cell growth in the capsules was inhibited at higher alginate concentrations due to an increased mass transfer resistance. This explanation appears to be more plausible in view of the relatively long-term inhibitory effect.

Yuet (1990) studied the diffusion of two growth-limiting nutrients, glucose and glutamine, through sodium alginate solution. He found that the viscosity of the solution (which increases with alginate concentration) had a remarkable influence on the diffusivity of glucose. When the viscosity increased from 20 to 50 cSt, the diffusivity of glucose was decreased to 8% of that in water. Yuet's results suggest that a high polymer concentration, which usually results in a high viscosity, may play a critical role in hindering nutrient mass transfer. Studies by others also support the hypothesis that there is a retarding effect on nutrient mass transfer in polymer solution. In particular, published reports on cell growth in microcapsules showed that the cells appeared to grow preferentially near the interior membrane surface of microcapsules (Posillico, 1986; King *et al.*, 1987).

Concluding remarks

While immobilization technology has been successfully applied to large-scale production of numerous anchorage-dependent and non-anchorage-dependent mammalian cells and their associated products, its application to insect cell culture is still very limited. For insect cell immobilization, the bottle neck is perhaps the lytic production process of non-secreted viral and

recombinant products, which prohibits extended retention of the immobilized catalysts in a production system. This barrier can only be removed with the development of alternative production processes, i.e., nonlytic, and secreted products. Moreover, the exploitation of anchorage-dependent cell lines may also make cell immobilization more advantageous. Nevertheless, immobilized cell culture has many advantages over suspension culture, such as significantly higher cell concentrations, and productivity, and simplified downstream processing. For full utilization of its advantages, though, there are several areas that need to be further explored, including suitable immobilization materials (i.e., biocompatibility and low cost), simpler immobilization techniques amenable to large-scale manufacture, as well as a good understanding of the mass transfer to and/or through the immobilization particles.

References

Agathos SN, Jeong Y-H and Venkat K (1989) Growth kinetics of free and immobilized insect cell cultures. Annals N.Y. Acad. Sci., 97, 372–398.

Butler M (1987) Growth limitations in microcarrier cultures. In: A Feichter (ed.), Adv. Biochem. Eng./Biotechnol., Springer-Verlag, Berlin, vol. 34, pp. 58–94.

Croughan MS, Hamel J-FP and Wang DIC (1988) Effect of microcarrier concentration in animal cell culture. Biotechnol. Bioeng., 32, 975–982.

Dean RC Jr, Karkare SB, Ray NG, Runstadler PW Jr and Vankatasubramanian K (1987) Large-scale culture of hybridoma and mammalian cells in fluidized bed reactors. Annals N.Y. Acad. Sci., 506, 129–146.

Glacken MW, Fleischaker RJ and Sinsky AJ (1983) Large-scale production of mammalian cells and their products: Engineering principles and barriers to scale-up. Annals N.Y. Acad. Sci., 413, 355–372.

Goosen MFA (1993) Insect cell immobilization. In: MFA Goosen, AJ Daugulis and P Faulkner (edss), Insect Cell Culture Engineering, Marcel Dekker, Inc., New York, N.Y, pp. 69–103.

Guo Y, Lou F, Peng Z and Korus RA (1990) Kinetics of growth and amylase production of immobilized Bacillus subtilis in an air-lift bioreactor. Biotech, Bioeng., 35, 99–102.

Heath C and Belfort G (1987) Immobilization of suspended mammalian cells: analysis of hollow fibre and microcapsules bioreactors. In: A. Feichter (ed) Adv. Biochem. Eng./Biotechnol., Springer-Verlag, Berlin, vol. 34, pp. 1–31.

Knazek RA, Gullino P, Kohler PO and Dedrick RL (1972) Cell culture on artificial capillaries. Science, 178, 65–67.

King GA, Daugulis AJ, Faulkner P and Goosen MFA (1987) Alginate-polylysine microcapsules of controlled membrane molecular weight cutoff for mammalian cell culture engineering. Biotechnol. Prog. 3, 231–240.

King GA, Daugulis AJ, Faulkner P, Bayly D and Goosen MFA (1988) Growth of baculovirus-infected insect cells in microcapsules to a high cell and virus density. Biotechnol. Lett., 10, 683–688.

King GA, Daugulis AJ, Goosen MFA, Faulkner P and Bayly D (1989) Alginate concentration: a key factor in growth of temperature-sensitive baculovirus-infected insect cells in microcapsules. Biotechnol. Bioeng., 34, 1085–1091.

Kompier R, Tramper J and Vlak JM (1988) A continuous process for the production of baculavirus using insect cell cultures. Biotechnol. Letters, 10, 849–854.

Lee DW, Piret JM, Gregory D, Haddow DJ and Kilburn DG (1992) Polystyrene macroporous bead support for mammalian cell culture. Annals N.Y. Acad. Sci., 665, 137–145.

Levine DW, Wong JS, Wang DIC and Thilly WG (1977) Somatic Cell Genetics, 3, 149.

Lim F and Moss R (1980) Microencapsulation of living cells and tissues. J. Pharm. Sci., 70, 351–354.

Luckow VA and Summers MD (1988) Trends in the development of baculovirus expression vectors, Bio/Technology, 6:47–55.

Park S and Stephanopoulos G (1992) Packed bed bioreactor with porous ceramic beads for animal cell culture. Biotechnol. Bioeng., 41, 25–34.

Pharmacia Fine Chemicals (1981) Microcarrier cell culture Principles & Methods, Box 175, S–75104 Uppsala 1, Sweden.

Posillico EG (1986) Microcapsulation technology for large-scale antibody production, Bio/Technology, 4(2), 114–117.

Reiter M, Blüml G, Gaida T, Zach N, Unterluggauer F, Doblhoff-Dier O, Noe M, Plail R, Huss S and Kattinger H (1991) Modular integrated fluidized bed bioreactor technology. Bio/Technology, 9, 1100–1102.

Reiter M, Blüml G, Zach N, Gaida T, Kral G, Assadian A, Schmatz C, Strutzenberger K, Hinger S and Kattinger H (1992) Monoclonal antibody production using the porous glass bead immobilization technique. Annals N.Y. Acad. Sci., 665, 146–151.

Reuveny S (1985) Microcarriers in cell culture: structure and application. In: K Maramorosch (edd), Adv. Cell Culture, Academic Press, Inc., Olando, Fla. vol 4, pp. 213–247.

Smith NA, Goosen MFA, King GA, Faulkner P and Daugulis AJ (1989) Toxicity analysis of encapsulation solutions and polymers in the cultivation of insect cells. Biotechnol Letters, 3(1), 61–66.

van Wezel AL (1967) Growth of cell-strains and primary cells on microcarriers in homogeneous culture. Nature, 216, 64–65.

Vaughn JL (1976) The production of nuclear polyhedrosis viruses in large-volume cell culture, J. Invertebre. Pathol., 28, 233–237.

Wickham TJ and Nemerow GR (1993) Optimization of growth methods and recombinant protein production in BTI-Tn–5Bl–4 insect cells using the baculovirus expression system. Biotechnol. Prog., 9, 25–30.

Weiss SA, Smith GC, Kalter SS and Vaughn JL (1981) In Vitro, 17, 495–502.

Wu J, King G, Daugulis AJ, Faulkner P and Goosen MFA (1992) Recombinant protein production in insect cells infected with a temperature-sensitive baculovirus. Cytotechnol., 9, 141–147.

Yuet P (1990) Mathematical modeling of animal cell growth in microcapsules. M.Sc. Thesis, Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada.

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Modelling baculovirus infection of insect cells in culture

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Key words: baculovirus, insect cells, mathematical model, population balance

Introduction

Mathematical modelling is a structured thought process using well-established principles of physical and biological science that emphasises quantitative rather than qualitative aspects of science. In the biological sciences, modelling complements experimentation by identifying significant parameters through the iterative process of making assumptions, formulating a model, and testing the model. The resulting mathematical model constitutes a compact means of expressing often complex mechanisms. The model can generally be used to interpolate between experimental observations and thus minimise the experimental effort required. Moreover, models can be used to extrapolate or predict the behaviour at previously unstudied parameter values, thus identifying new research area of interest and forming a null hypothesis for experimentation.

Review of the literature shows that only three comprehensive mathematical models have been developed to describe the infection of insect cells with baculovirus:

1. Licari & Bailey (1992) modelled β-galactosidase production using a recombinant AcNPV to infect Sf9 cells in static batch cultures,
2. de Gooijer *et al.* (1989+1992) modelled production of wild type AcNPV virus by Sf9 cells in a continuous cultivation system consisting of one upstream bioreactor, in which insect cells are cultured, followed by one or more serially linked bioreactors where infection takes place, and
3. Power & Nielsen (Power *et al.*, 1992; Nielsen *et al.*, 1994; Power *et al.*, 1994) modelled β-galactosidase production using a recombinant AcNPV to infect Sf9 cells in batch suspension cultures.

The reason for this limited number of models can presumably be attributed to the fact that there is no trivial entry level model. In cell only systems, Monod type models provide a good starting point for a description of the system. Cell-virus systems are too complex to be described by such simple models. This complexity, however, is also one of the reasons why modelling is so important in cell-virus systems. Without a model it is almost impossible to interpret some experimental results, for example:

- the behaviour of an insect cell culture infected at low multiplicity where non-infected cells and cells in different stages of infection coexist at any given point of time, and
- the accumulation of defective interfering particles (DIPs) in continuous cultivation (Kool *et al.*, 1991; Wickham *et al.*, 1991).

The key difference between traditional cell only models and models of cells infected with a lytic virus is that cells infected with a lytic virus will during the infection go through a characteristic set of events – the infection cycle. During the infection cycle the cell metabolism totally changes from producing biomass to producing viral products. This temporal change in cell metabolism must be built into the model. A model of the baculovirus-insect cell interaction includes several elements:

- a model of non-infected cell growth,
- a model of the physical interaction between virus and cells (binding and infection),
- a model of the infection cycle in individual cells,

- a population balance model to take into account that cells may be infected at different points in time and thus at any point in time the cumulative kinetics of a culture is a combination of the kinetics shown by cells in various stages of infection,
- a mass balance model to take into account the physical system, e.g. describing substrate consumption in a batch culture or the dilution effect in a continuous culture.

The three existing models incorporate these elements in slightly different ways reflecting the key parameters in the different systems considered. In this paper, we will ignore minor differences in order to highlight the common elements required to model baculovirus-insect cell interaction and only highlight the few significant differences between the models. Taking this approach it is our hope to provide newcomers to the area with a clearer picture of the modelling process and the assumptions made than is normally feasible within the scope of a research paper.

Non-infected cell growth

Cell growth before infection could be described by any traditional growth model. Experience tells us, however, that cells must be infected in the mid-exponential phase in order to achieve a reasonable product yield. Thus, all relevant situations can be modelled with a simple exponential growth model

$$\frac{dN_V}{dt} = (\mu_{max} - k_D)N_V - IR(t), \qquad (1)$$

where N_V is the concentration of viable cells, μ_{max} the maximum specific growth rate, k_D the specific death rate, and IR the infection rate (see later). The non-infected cell parameters, μ_{max} and k_D, can be determined from viable and dead cell numbers during the exponential phase of a non-infected culture.

The infection cycle

Following virus attachment and internalisation, the virus-cell system progresses through a series of characteristic events, the infection cycle. The main contribution to our understanding of these events has come from empirical studies of insect cell physiology and both *in vivo* and *in vitro* baculovirus replication (e.g. Granados, 1980; Volkman & Knudson, 1986; Faulkner & Carstens, 1986). The fundamental understanding of baculovirus replication afforded by these studies serves as a sound basis on which mathematical models can be formulated.

There are two approaches that can be taken to describe the infection cycle, an unstructured and a structured approach. In the unstructured approach the temporal element of the infection cycle is described by a number of time varying rates. No attempt is made to follow the internal events of the cell. The three existing models all employ this approach.

In the structured approach, biomass is divided into pools (cell pools, viral DNA, viral RNA, virus specific DNA and RNA polymerase, etc.) and the dynamic behaviour of these pools is described by rate expressions in terms of the pool sizes. At present no working structured model exists, though Shuler *et al.* (1990) outlined a possible structure for such a model.

Unstructured models

In unstructured models, the independent parameter used to account for the temporal development of the infection cycle is time since infection, τ. In the Licari & Bailey and the de Gooijer models, there is also a distinction between the type of infection experienced by the cell.

The model of Licari & Bailey (1992) considers in addition to the time since infection, the effect of the multiplicity of infection. The latter results in 10 different infected cell populations distinguished by the number of viruses infecting the individual cell. The cell populations differ in that the timing of infection events is different, while the total yields of virus and product are constant.

To accommodate the passage effect, de Gooijer *et al.* (1992) developed a model in which three different NOV types where produced in infected cells: normal infective particles (I-NOVs), particles which lead to an abortive infection (A-NOVs), and defective interfering particles that lack about 44% of the virus genome (D-NOVs). The three virion types led to three infection modes and thus three types of infected cell populations. The first mode is a correct infection arising from entry of at least one I-NOV, without any D-NOVs. This infection gives rise to the production of I-NOVs, A-NOVs, and a small number of D-NOVs. The second mode of infection arises from simultaneous infection of a cell by at least one I-NOV and at least one D-NOV. The genetic advantage of the D-NOV results in production of large quantities of D-NOVs and few of the other two

virion types. The third mode results from infection of a cell with an A-NOV and/or a D-NOV in the absence of an I-NOV. Without the helper effect of the I-NOV, such infections do not produce any virions.

In order to illustrate the unstructured infection cycle models, we shall here only consider the simplest situation with a single type of infected cell population. This was the approach taken by Power *et al.* (1994) to describe β-galactosidase production using a recombinant AcNPV to infect Sf9 cells in suspension culture.

The infection cycle of wildtype baculovirus can be divided into three phases (Miller, 1988): early (0–6 hours post infection), late (8–18 hours post infection), and very late (20–72 hours post infection). The late phase is characterised by the synthesis of nonoccluded virions capable of budding from the cell surface. The very late phase is characterised by the embedding of virions in occlusion bodies composed primarily of polyhderin, a 29-kD protein produced in abundance during the very late phase. For modelling purposes, the time points of interest are those linked with readily observable events:

- virus release,
- staining of cells,
- β-galactosidase production,
- β-galactosidase release.

Extracellular virus is not observed in the culture fluid until around the onset of the very late phase. Electron microscopy observations (unpublished) have confirmed that virus progeny is produced from the beginning of the late phase, τ_E. The newly produced virus progeny was observed on the cell surface and some was observed to readsorb to the cell of origin. Surface presentation of non-occluded virus in the late phase could be the means by which lateral infection of proximal cells occurs in the gut cells of insects (Granados & Williams, 1986). It is not clear if the release rather than surface presentation of virus progeny is linked to the very late phase or simply is a delayed process. Hence, in all three models the commencement of virus release is designated by its own marker, here designated τ_{VRC}. Virus appears to be released at a relatively constant rate, α_{VR}, and ends after the end of the very late phase at a point designated, τ_{VRE}. Thus, the specific rate equation for virus release, r_{VR}, is

$$r_{VR}(\tau) = \begin{cases} 0 & , \text{for } \tau \leq \tau_{VRC} \\ \alpha_{VR} & , \text{for } \tau_{VRC} < \tau \leq \tau_{VRE} \\ 0 & , \text{for } \tau \geq \tau_{VRE} \end{cases} . \quad (2)$$

Cell viability of infected cells are not considered in any of the three models. Trypan blue dye exclusion is a measure of membrane integrity. For non-infected cells, membrane integrity can be used as an indicator of viability. For infected cells, however, membrane integrity gradually decreases during the late part of the infection rather than spontaneously at the end of infection (Licari & Bailey, 1992). As virus and recombinant product are released gradually as well, there is no particular need for the model to identify the point of death and lysis. The gradual staining of cells is expressed with an empirically fitted equation giving the fraction of stained cells as a function of τ

$$t_U(\tau) = \begin{cases} 1 & , \tau \leq \tau_U \\ e^{-\beta_U(\tau - \tau_U)} & , \tau > \tau_U \end{cases} , \quad (3)$$

where τ_U is time following infection where staining commences and β_U is the first order rate of staining hereafter. Using a similar approach, de Gooijer *et al.* (1992) employed the gradual visual changes in infected cells to follow the progression of infection with wildtype virus.

In the baculovirus expression vector system, recombinant protein is expressed under the control of the polyhedrin promoter. Thus, recombinant protein expression is directly linked to the expression of the very late genes commencing at τ_L and terminating at τ_{VL}. The production rate is assumed constant, α_{PP}. Both intracellular and extracellula β-galactosidase can be determined experimentally. β-galactosidase is not a secreted product and protein release is assumed to be linked to the leakiness of the membrane as evidenced by trypan blue uptake. Thus, release is assumed to commence at the same time as staining, τ_U. Hereafter, the rate of release is assumed to be proportional to the intracellular concentration (the release constant denoted α_{PR}), i.e.

$$r_{PR}(\tau) = k_{PR}(\tau)p_i(\tau), \quad (4a)$$

where

$$k_{PR}(\tau) = \begin{cases} 0 & , \text{for } \tau < \tau_U \\ \alpha_{PR} & , \text{for } \tau \geq \tau_U \end{cases} , \quad (4b)$$

The intracellular concentration, p_i, is given by

$$\frac{dp_i}{d\tau} = r_{PP}(\tau) - r_{PR}(\tau)p_i; p_i(0) = 0, \quad (5a)$$

where $r_{PP}(\tau)$ is the specific productivity

$$r_{PP}(\tau) = \begin{cases} \alpha_{PP} & \text{for } \tau_L \leq \tau \leq \tau_{VL} \\ 0 & \text{otherwise} \end{cases}, \qquad (5b)$$

Solving Equation (5), the intracellular concentration can be determined

$$p_i(\tau) = \begin{cases} 0 & \text{for } \tau \leq \tau_L \\ \alpha_{PP}(\tau - \tau_L) & \text{for } \tau_L < \tau \leq \tau_U \\ \frac{\alpha_{PP}}{\alpha_{PR}} + \alpha_{PP}\left(\tau_U - \tau_L - \frac{1}{\alpha_{PR}}\right) e^{-\alpha_{PR}(\tau-\tau_U)} & \text{for } \tau_U < \tau \leq \tau_{VL} \\ p_i(\tau_{VL} e^{-\alpha_{PR}(\tau-\tau_{VL})} & \text{for } \tau > \tau_{VL}. \end{cases} \qquad (6)$$

The number of parameters in the above model may initially seem large. All parameters, however, can be determined and visually verified using a synchronous infection of cells in the midexponential phase. If a batch culture of insect cells is infected at a high multiplicity (MOI>5), the ensuing infection process will be essentially synchronous, i.e. all cells will go through the infection cycle simultaneously. Accordingly, the biomass can be treated as non-distributed or non-segregated. In other words, the total rates observed in a synchronous culture is simply the total number of cells multiplied with the specific rates outlined above evaluated in τ equal time since infection. The three β-galactosidase production parameters: onset of production (τ_L), end of production (τ_{VL}), and the constant rate of production between these points (α_{PP}), for example, can be readily fitted and verified by plotting the total β-galactosidase concentration on a per cell basis versus time post infection (Figure 1).

Structured models

It is likely that we will not be able to adequately describe all the complex interactions in the system until we begin modelling what goes on inside the cell following infection, i.e. until we shift towards structured models. One problem that definitely calls for a structured modelling approach is the formation of multi subunit proteins by simultaneous use of two or more recombinant viruses.

Although structured models hold large promises in terms of gaining a quantitative understanding of the mechanisms of baculovirus infection, it is important to understand their limitations. The key to developing a good structured model is to obtain good measurements of the intracellular pools. Without such measurements the rate equations and parameters can not be validated and are likely to become nothing but a sophisticated fitting machine.

The complexity of structured models can also become prohibitive in terms of numerically solving problems such as low multiplicity infection. Combining a rich structured model of what happens in the individual cell following infection with a population balance model describing the various times cells are infected is by no means easily done. Hence, low MOI infections will most likely continue to be described by the unstructured approach outlined below.

Population balance for asynchronous infection

When cells are infected at an MOI less than 3–5 in a batch culture, the culture will no longer be synchronous. At any point in time, the culture will be composed of non-infected cells and cells at different points in their individual infection cycle. The culture behaviour is the combined behaviour of these individual cells. In a continuous culture non-infected cells are added continuously and the culture will obviously be asynchronously infected.

In order to keep track of the culture, we introduce the cell density function for infected cells, $\mathbf{n(t,\tau)}$, where t is true time and τ is time since infection. Note that $\mathbf{n(t,\tau)d\tau}$ represents the number of cells at true time t, that have been infected for a period between τ and $\tau + \mathbf{d\tau}$ hours. Assuming no post infection growth the law of conservation for cell numbers reads

$$\begin{pmatrix} \text{change in number} \\ \text{of cells in } [\tau,\ \tau + \mathbf{d\tau}] \end{pmatrix} =$$

$$\begin{pmatrix} \text{number of cells} \\ \text{entering } [\tau,\ \tau + \mathbf{d\tau}] \end{pmatrix} -$$

$$\begin{pmatrix} \text{number of cells} \\ \text{leaving } [\tau,\ \tau + \mathbf{d\tau}] \end{pmatrix}$$

$$n(t + dt,\ \tau)d\tau - n(t,\ \tau)d\tau =$$

$$\nu(t,\ \tau)n(t,\ \tau)dt - \nu(t,\ \tau + d\tau)n(t,\ \tau + d\tau)dt$$

$$\frac{\partial n}{\partial t} + \nu\frac{\partial n}{\partial \tau} = 0. \qquad (7)$$

The maturation velocity, ν, for this system is one hour of infection time per one hour of true time. Normally, it will be assumed that $\mathbf{n(0,\tau) = 0}$, i.e. at t = 0 no cells are infected. If furthermore the infection rate at all times

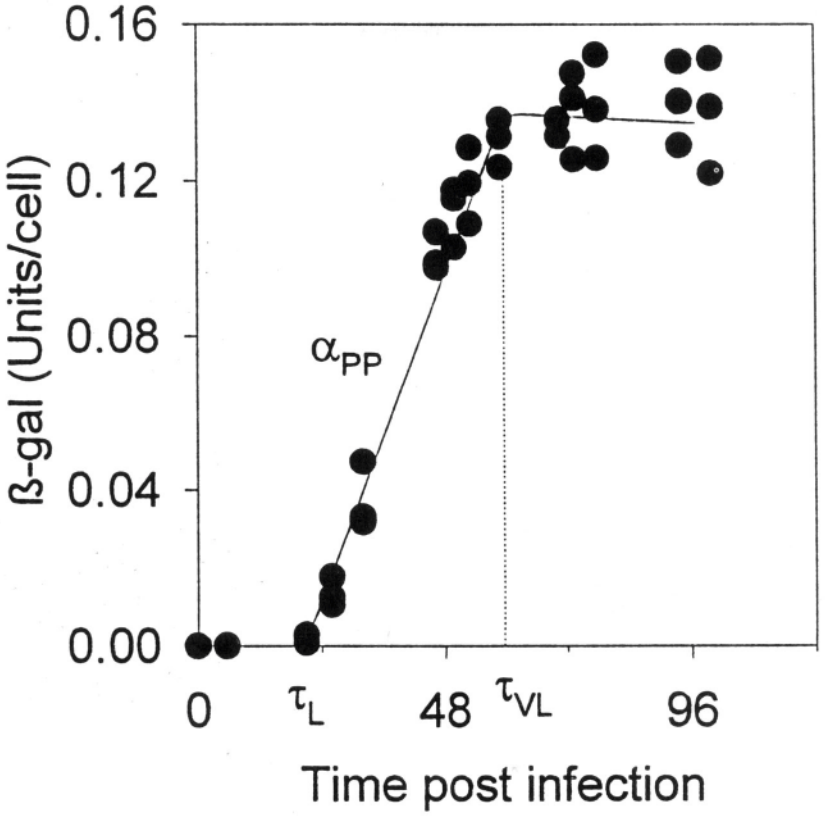

Figure 1. Fitting β-galactosidase production parameters. The three β-galactosidase production parameters: onset of production (τ_L), end of production (τ_{VL}), and the constant rate of production between these points (α_{PP}), are fitted from a series of high multiplicity infection experiments (see Power *et al.*, 1994, for details).

t, n(t,0), is specified, then Equation (7) has a unique solution.

The assumption of no post infection growth made by all three existing models is not absolutely true. In our experience, the total number of cells does increase in average 15% post infection, suggesting that cells may continue to divide through the early phase of infection. Accounting for this growth, however, would involve deciding whether both daughter cells in a division are infected or only one of them. It is possible to determine this based on the multiplicity of infection, but introducing this distinction.adds substantially to the numerical effort.

Mass balances

Mass balances are formulated by combining the population balance model with the cell cycle model. A mass balance for extracellular β-galactosidase, for example, leads to the following ordinary differential equation

$$\frac{dP_{EX}}{dt} = \int_{\tau=0}^{\infty} r_{PR}(\tau) n(t, \tau) d\tau - k_{DP} P_{EX}, \quad (8)$$

where the integral term accounts for the release from cells at different stages of infection and the second term accounts for a first order decomposition of the product.

The simple model as described so far provides a reasonably good description of low multiplicity experiments (Figure 2). It should be stressed that all parameters in the model were determined from high MOI experiments. Still, the model was capable of describing the behaviour of the system at a multiplicity 5 magnitudes lower. In other words, low multiplicity infections in which only a fraction of cells is infected initially behave in a manner that can be predicted by the behaviour at high multiplicity infections.

If several population types are considered as in the case of Licari & Bailey's model, the resulting mass

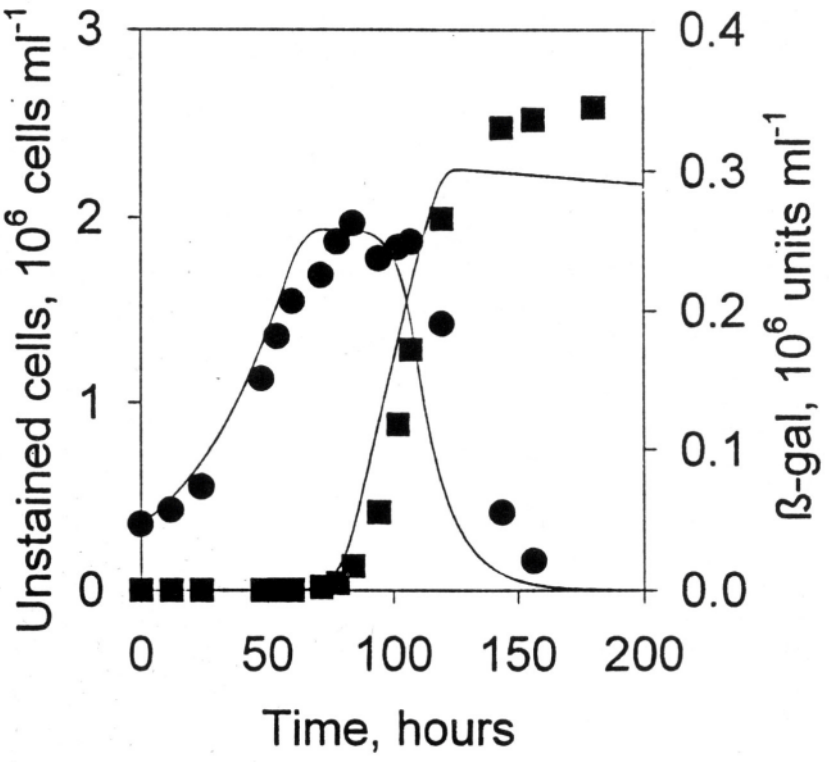

Figure 2. Predicting low multiplicity batch infection in suspension culture. A 1 L bioreactor culture at 0.35×10^9 cells dm^{-3} was infected at a multiplicity of 0.0001. The figure depicts the observed and predicted unstained cell density (circles) and total β-galactosidase (squares) (unpubl.).

balance involves a sum of integrals for each population, such as

$$\frac{dP_{EX}}{dt} = \sum_{i=1}^{\text{No. populations}} \int_{\tau=0}^{\infty} r_{PR,i}(\tau) n_i(t, \tau) d\tau - k_{DP} P_{EX}, \tag{9}$$

where n_i is the cell density function for the i'th population and $r_{PR,i}$ is the corresponding release function.

System limitation

It is well established that successful expression of recombinant product only occurs if the batch culture is infected before the late exponential/stationary phase. This key limitation of the system was in the Licari & Bailey model of static cultures described as spatial limitation and the production rates of the system was set proportional to the fraction free space, A^*

$$A^* = \frac{\text{total capacity} - \text{capacity taken by noninfected and infected cells}}{\text{total capacity}}. \tag{10}$$

In suspension cultures, the limitation appears to be related to the depletion of an essential substrate (Lindsay & Betenbaugh, 1992; Reuveny *et al.*, 1993). The de Gooijer model ignored this limitation, in essence assuming that the cell only reactor upstream was operated such that sufficient nutrients were carried over into the downstream infection reactor.

The Power & Nielsen model had to overcome several problems in order to describe substrate limitation:

1. *Limiting substrate is unknown.* The limiting substrate is still to be identified and the model had to assume an arbitrary substrate to be present at 1 Unit per litre of fresh medium.
2. *Substrate limitation causes complex changes in kinetics.* It has been our experience that substrate limitation causes a general slow down of the growth

and infection processes, not only a lowering in production rates (as assumed by Licari & Bailey, 1992). As a simple initial approach, the model was made a simple depletion model, i.e. all processes were assumed to occur at maximum rate until depletion of substrate, at which point all processes would stop.

3. *Consumption pattern unknown.* The limiting substrate being unknown it was not possible to formulate a consumption profile. Instead the specific consumption rate was assumed constant for noninfected and infected cells. The concept was that substrate consumption remained constant while the end-product would change from cell mass to viral products over the infection.

With these simplifying assumptions the resulting substrate limitation model only required a single parameter to be obtained, namely the maximum cell density observed in a noninfected culture.

Despite its simplicity the model is reasonably successfully in predicting the total product titre observed when cells are infected at different points during a batch (Figure 3). The model predicts the initial increase in product titre as the cell density at time of infection increases. It also predicts the peak in yield in the mid-exponential phase and the following decline in yield. The model does not predict the small amount of β-galactosidase produced by cells infected late in the exponential phase. For practical purposes, however, the model predicts the important peak in yield.

While the model predicts the final β-galactosidase concentration beyond the peak in yield, the temporal development is not well described around the peak (not illustrated). This difference may be attributed to the failure of the model to describe the slow down in growth and infection when substrate becomes limiting. One possible solution to this inadequacy is to make maturation velocity, ν, a function of substrate concentration, e.g.

$$\nu(S) = \frac{S}{K_s + S}. \qquad (11)$$

Note, that with this definition the "infection time", τ, becomes a marker of the progression through the infection cycle rather than a measure of time. At $S = K_S$, for example, the cell progress half an "infection hour" for each true hour in culture. If all specific rates also are multiplied with ν, the result is to stretch out the infection cycle at lower substrate levels without changing the tolal yields.

This approach differs from that used by Licari & Bailey, in that they only took the change in production rale into account, not the prolonging of the infection cycle. The new approach is yet to be tested. The constant K_S will have to be a fitted constant, i.e. chosen as the value that gives the best description to a series of high MOI experiments infected at different initial cell density. From a numerical point of view, the approach is a non-trivial extension of the model. With a constant maturation velocity of 1, the partial differential Equation (7) has the simple solution

$$n(t, \tau) = n(t - \tau, 0). \qquad (12)$$

whereas with a time varying maturation velocity, the equation must be solved numerically.

Binding and infection

The first event of the infection cycle is the physical binding of virus to cells. Binding differs from infection in that the cells continue to bind further virus long after the initial infection has occurred. Experimentally, we have observed binding for at least 8 hours post infection and binding may occur until 15–20 hours post infection when virus release commences.

Binding and internalisation are important processes in order to understand the possible effect of multiple binding, the formation of multi-subunit proteins, and the formation of DIPs. None of the three models presented provides a satisfying description of this aspect of the system. The Power & Nielsen model acknowledges the difference between binding and infection, but does not incorporate any account of the effect of multiple infection. The Licari & Bailey model and de Gooijer's model both consider the distribution of virus particles on cells, but only within a single discretisation step, thus ignoring the temporal element of virus binding. The limitation of the three models will be discussed in detail after this short description of the issues concerning binding and infection.

The issues

The initial binding process has recently been under some scrutiny. In the work of Wickham *et al.* (1990), the binding of viruses to receptors on cells was studied in detail for a number of different animal viruses. Three modes of virus attachment were proposed:

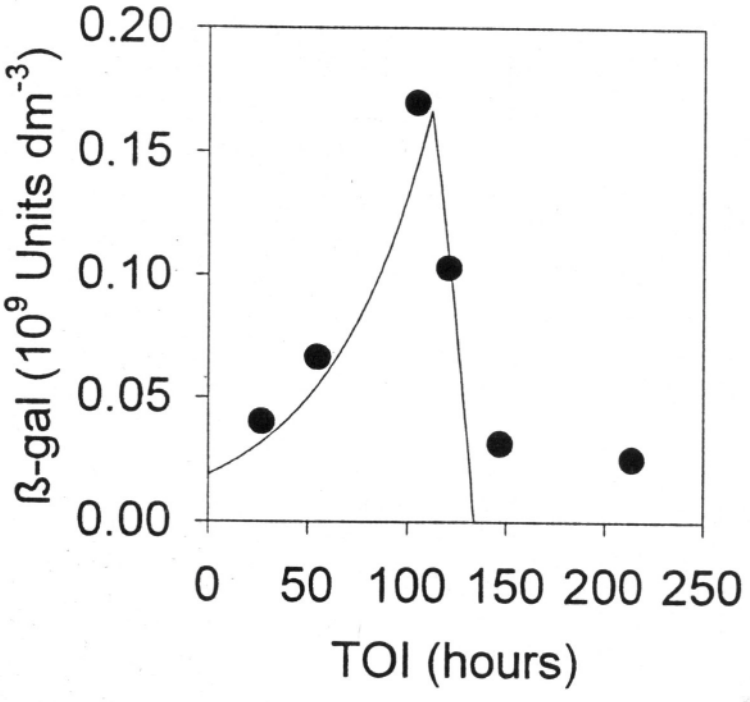

Figure 3. Limitation in batch suspension culture. 100 mL cell aliquots were removed from a batch culture of sf9 cells, infected at a MOI of 10, and the infected culture placed in an incubator. The figure shows the maximum titre observed in the ensuing infection, together with the predicted maximum titre (see Power *et al.*, 1994, for details).

Mode 1 involved single virus binding to a single receptor.

Mode 2 occurred when a virus first attached to a single receptor then initiated a series of reversible binding reactions with other receptors on the cell surface.

Mode 3 was the same as mode 2 but it included spatial saturation.

The third mode of virus attachment was proposed for cells with very high numbers of viral receptors which become completely covered with virus before the receptors are saturated. It was hypothesised that baculoviruses attach to insect cells via this mode.

More recently, Wickham *et al.* (1992) found that for *T. ni* cells the number of binding sites determined from equilibrium binding were high enough to indicate an involvement of spatial saturation. The number of binding sites on *S. frugiperda,* however, is only 15-25% of that for *T. ni* – ~3000 binding sites per cell (Wickham *et al.*, 1992; Granados, 1994) – and spatial saturation may not be limiting factor.

These studies were performed at low temperature where internalisation and infection is minimal. Knowing that the cell can possibly bind 3000 viruses at any point in time – and possible more over time as virus is internalised – raises many questions regarding the initial infection process:

- How many viruses can actually be internalised and what is the kinetics of this process?
- How does the number of virus particles internalised affect the ensuing infection process?
- How long after the initial internalisation of virus will further internalisation affect the infection process?
- Does the remaining virus temporarily bind to infected cells without being internalised and if so for how long?

Answering these and similar questions is likely to take years of dedicated experimental and analytical effort.

Licari & Bailey model

Licari & Bailey (1992) suggested, based on unpublished observations, that the timing of events in the infection cycle is strongly dependent on the number of virions infecting a given cell. In our view, it is not

totally clear whether the spreading out of the infection response at lower multiplicities is due to a slow down in the infection cycle or simply a spreading out of the initial infection process. Conceptionally, the observation of Licari & Bailey makes sense. If more viral material is available initially, the infection should be faster at least with respect to viral genome replication. Assuming Licari & Bailey's hypothesis is correct, a need arises to know not only when cells have been infected but also with how many virions the cells have been infected.

Licari & Bailey first considered the initial infection situation where a monolayer of cells is overlayed for 1 hour with a medium containing virions. By direct analogy to a haemocytometer, they observed that – given infinite contacting time – the number of virions infecting an individual cell would be described by a Poisson distribution with mean equal the multiplicity of infection (MOI), i.e. the ratio of the number of virions to the number cells. In reality, the contacting time of 1 hour is too short for all virions to be distributed on the monolayer of cells and when the inoculum medium is withdrawn negligible depletion of virus was observed. To account for this, Licari & Bailey introduced a proportionality factor, α, accounting for what they termed the *physical system.* Hence, the number of virions infecting an individual cell was described by a Poisson distribution with mean α-MOI.

Based on the above consideration, Licari & Bailey continued to propose that *at any time* in this system the probability of NV virions infecting a cell, P(NV,t), would be Poisson distributed:

$$P(NV,t) = P\{\text{Poisson}(\alpha\cdot\text{MOI}) = \text{NV}\}. \quad (13)$$

This is obviously wrong. The situation now considered has changed from one of infections occurring over a period of time (the contacting time) to that of instantaneous infection. The contacting time is obviously an extremely important factor in determining α, the fraction of virions bound. The situation of instantaneous infection may be described by a Poisson process with a rate of occurrence given by

$$\rho = \frac{\alpha\cdot\text{MOI}}{\text{contacting time}}. \quad (14)$$

Only when sampled for a period of time, h, does the Poisson distribution arise. The difference is important. A probability for one or more infections to occur can not be assigned for the instant; it can only be assigned for a period of time. If assigned to the instant repeating the assignment for the following couple of "instants" would see all virus distributed within a couple of "instants", that is instantaneously.

It would appear, that Licari & Bailey applied Equation (13) to the discrete interval arising when solving the full model numerically and thus indirectly introduced a contacting time. According to their paper, they used a step size of 1 hour, which would correspond to the initial contacting time used to determine α. Although, it is theoretically correct to consider multiple infection in discrete time intervals, it is not clear what biological significance an arbitrarily chosen discretisation into one hour intervals has. The questions arise of how long after the initial infection of a cell does further infections affect the infection behaviour and to what degree.

Another question is whether the physical system really is the same. The reason for the small fraction binding in the initial 1 hour contacting time in a static culture is likely to be that the diffusivity of virus is low and much virus will never reach the cells. In secondary infection, virus is released from the monolayer and the path to proximate cells is shorter and the binding rate could be higher.

Finally, the assumption that only non-infected cells can bind virus does not agree with the observation made above, that cells continue to bind virions at least 6–8 hours (and possible as long as 15–20 hours) after initial infection.

De Gooijer model

De Gooijer's model considered three modes of infection and thus it was necessary to consider the multiplicity of infection and the relative distribution of the three different types of NOV. Using a similar approach as Licari & Bailey, it was assumed that infection would occur within one time step and that infected cells would not bind further virus. In the well-mixed suspension culture, it was assumed that limitation in binding was due to a limited number of binding sites. All the available virus would spread over 30–40 active binding sites with equal affinity for all three types of NOV. The probability of each type of infection was calculated for each time interval given the concentration of the different NOV types.

The number of binding sites is a fitted constant which is much less than the measured value of ~3100 (Granados, 1994). This fitted constant is a composite constant indicating not only how many viruses bind,

but also how many are internalised and establish a successful infection. It is possible that more viruses bind and that some viruses are internalised too late to be replicated. As for the Licari & Bailey model, the kinetics of multiple binding and internalisation remains to be studied. Virus binding kinetics could be important in the dynamics of continuous cultures, as temporary binding to an infected cell may introduce a lag in the system and could result in a carry over from the first infection tank into the second in a dual infection tank configuration.

Despite the possible limitations in the virus binding model, the de Gooijer model has proven successful in fitting the DIP effect observed in continuous culture. Using this model, it was also possible to propose a repeated batch mode of operation, in which the DIP accumulation was greatly reduced. Recent work has shown that the repeated batch mode does lead to the predicted delay in DIP accumulation (van Lier *et al.*, 1996).

Power & Nielsen model

The Power & Nielsen model distinguishes between binding and infection. In the well-mixed environment of a suspension culture, the virus binding rate, BR(t), is proportional to the extracellular virus concentration (Power *et al.*, 1995). The virus binding rate was observed not to increase linearly with cell density. The decrease in virus binding efficiency with increasing cell density is presumably due to cell clumping. The following empirical function was found to describe attachment kinetics over a broad range of virus and cell concentrations

$$BR(t) = \alpha_B e^{-\beta_B N_{TOT}} N_B V, \qquad (15)$$

where α_B and β_B are fitted empirical constants, N_{TOT} the total number of cells, and N_B the number of virus binding cells (i.e. non-infected cells plus cells infected for less than 15–20 hours).

The Power & Nielsen model does not make a distinction between infected cells on the basis of the number of virions they bind. As mentioned above it is not clear whether multiple infection causes a significant change in the infection cycle. Ignoring the multiplicity effect, the infection rate, IR(t), simply is the binding rate for previously non-infected cells, i.e.

$$IR(t) = \frac{N_V}{N_B} BR(t) \frac{\text{cells}}{\text{PFU}} =$$

$$\alpha_B e^{-\beta_B N_{TOT}} N_V V \frac{\text{cells}}{\text{PFU}}, \qquad (16)$$

The correlations developed in Equations (15) and (16) are useful temporary measures while a proper quantitative description of the binding, internalisation and initialisation of infection is being developed. Unlike the models suggested by Licari & Bailey and de Gooijer, these correlations take the temporal element into account. The correlations do not, however, offer any means of describing the possible multiplicity effect or the DIP effect.

Conclusions

Infection of insect cells with baculovirus is a potentially attractive means for producing both viral insecticides and recombinant proteins. The continuation of mathematical modelling studies such as those reviewed in this paper are essential in order to realise the full potential of the system. Through mathematical models it is possible to predict complex behaviours such as those observed when infecting cells at low MOI or when propagating virus in a continuous culture system. A purely empirical analysis of the same phenomena is very difficult if not impossible.

The present three models are – despite their complexity and the effort that has gone into developing them – all first generation models. They summarise, to a large extent, our present quantitative understanding of the interaction between baculovirus and insect cells, when looked upon as a black box system. The binding and initial infection processes are still quantitatively poorly understood and further work in this area is much needed. On the longer term, a second generation of models is likely to consider interior processes such as viral DNA and RNA accumulation in much more detail using a structured model of the infection cycle.

References

Faulkner P & Carstens EB (1986) An overview of the structure and replication of baculoviruses. Curr. Top. Microbiol. Immunol. 131: 1-19.

de Gooijer CD, van Lier FLJ, van den End EJ, Vlak JM & Tramper J (1989) A model for baculovirus production with continuous insect cell cultures. Appl. Microbiol. Biotechnol. 30: 497–501.

de Gooijer CD, Koken RHM, van Lier FLJ, Kool M, Vlak JM & Tramper J (1992) A structured dynamic model for the baculovirus

infection process in insect-cell reactor configurations. Biotechnol. Bioeng. 40: 537–548.

Granados RR (1980) Infectivity and mode of action of baculoviruses. Biotechnol. Bioeng. 22: 1377–1405.

Granados RR & Williams KA (1986) *In vivo* infection and replication of baculoviruses. In Granados RR & Federici BA (eds) The Biology of Baculoviruses. Vol. 1 (pp. 89–108) CRC Press, Boca Raton, Florida.

Granados RR (1994) Presented at the ICIP conference, August, Montpellier, France.

Kool M, Voncken JW, van Lier FLJ, Tramper J & Vlak JM (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology 183: 739–746.

Licari P & Bailey JE (1992) Modeling the population dynamics of baculovirus-infected insect cells: optimizing infection strategies for enhanced recombinant protein yields. Biotechnol. Bioeng. 39: 432–441.

Lindsay DA & Betenbaugh MJ (1992) Quantification of cell culture factors affecting recombinant protein yields in baculovirus-infected insect cells. Biotechnol. Bioeng. 39: 614–618.

Miller LK (1988) Baculoviruses as gene expression vectors. Ann. Rev. Microbiol. 42: 177–199

Nielsen LK, Power J, Radford K, Reid S & Greenfield PF (1994) Modelling insect cell cultures infected with recombinant baculovirus. In Spier RE, Griffiths JB & Berthold W (eds) Animal Cell Technology: Products of Today, Prospects of Tomorrow (pp. 518–520) Butterworth-Heinemann, Oxford.

Power J, Greenfield PF, Nielsen LK & Reid S (1992) Modelling the growth and protein production by insect cells following infection by a recombinant baculovirus in suspension culture. Cytotechnology 9: 149–155.

Power JF, Reid S, Radford KM, Greenfield PF & Nielsen LK (1994) Modeling and optimization of the baculovirus expression vector system in batch suspension culture. Biotechnol. Bioeng. 44: 710–719.

Power JF, Reid S, Greenfield PF & Nielsen LK (1995) The kinetics of baculovirus adsorption to insect cells in suspension culture. Cytotechnology, In press.

Reuveny S, Kim YJ, Kemp CW & Shiloach J (1993) Production of recombinant proteins in high-density insect cell cultures. Biotechnol. Bioeng. 42: 235–239

Shuler ML, Cho T, Wickham T, Ogonah O, Kool M, Hammer DA, Granados RR & Wood HA (1990) Bioreactor development for production of viral pesticides or heterologous proteins in insect cell cultures. Ann. NY Acad. Sci. 589: 399–422.

Van Lier FLJ, van den Hombergh JPTW, de Gooijer CD, Den Boer MM, Vlak JM & Tramper J (1996) Long-term semi-continuous production of recombinant baculovirus protein in a repeated (fed-)batch two-stage reactor system. Enzyme Microb. Technol., 00: 000–000.

Volkman LE & Knudson DL (1986) In vitro replication of baculoviruses. In Granados RR & Federici BA (eds) The Biology of Baculoviruses. Vol. 1 (pp. 109–127) CRC Press, Boca Raton, Florida.

Wickham TJ, Granados RR, Wood HA, Hammer DA & Shuler ML (1990). General analysis of receptor-mediated viral attachment to cell surfaces. Biophysical Journal 58: 1501–1516.

Wickham TJ, Davis T, Granados RR, Hammer DA, Shuler ML & Wood HA (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnol. Lett. 13: 483–488.

Wickham TJ, Shuler ML, Hammer DA, Granados RR & Wood HA (1992) Equilibrium and kinetic analysis of *Autographa californica* nuclear polyhedrosis virus attachment to different insect cell lines. J. Gen. Virol. 73: 3185–3194.

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Scale up aspects of sparged insect-cell bioreactors

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Introduction

In small-scale cultures of insect cells, and animal cells in general, gentle agitation is required to keep the cells from settling and to provide a homogeneous environment. At larger scales, more vigorous agitation is needed in order to enhance the mass transfer rate of both nutrients and toxic metabolites. Besides more vigorous agitation, sparging might be required to increase the oxygen transfer rate. However, hydrodynamic forces associated with agitation and sparging can be detrimental to animal cells, which has led to the design of bubble-free aeration reactors (Henzler, 1993). Although these reactors have been operated succesfully, they are also limited in their application scale and usually have a more complex design than the standard reactors, like the stirred tank, bubble-column and air-lift loop reactor (Henzler, 1993). At larger scales the use of bubble-column and air-lift loop reactors might become feasible, in addition to the conventional stirred-tank reactor. The former reactors have, even at larger scales, good oxygen and mass transfer characteristics, and a relative simple design through the absence of mechanical agitation. Moreover, the hydrodynamic behaviour and mass transfer characteristics are well documented in literature (Chisti, 1989). However, as stated, the presence of air bubbles may cause cell damage and death.

The effect of hydrodynamic forces generated by air bubbles on suspension cells has been extensively studied (Tramper *et al*., 1986; Tramper *et al*., 1988; Handa-Corrigan *et al*., 1989; Kunas & Papoutsakis, 1990; Van der Pol *et al.,* 1990; Bavarian *et al*., 1991;Chalmers & Bavarian, 1991; Jöbses *et al*., 1991; Cherry & Hulle, 1992; Martens *et al*., 1992; Martens *et al*., 1993; Jordan *et al*., 1994; Trinh *et al*., 1994), as well as the effect of agitation on microcarrier cultures (Cherry & Papoutsakis, 1986; Croughan *et al*., 1989; Croughan & Wang, 1990; Lakothia & Papoutsakis, 1992), which was reviewed by Papoutsakis (1991). In this chapter the various aspects are discussed with respect to scale up.

Shear

Any cell, when placed in a moving fluid with velocity gradients, experiences a shear force which magnitude depends on the dynamic viscosity of the fluid, the fluid velocity gradients, and the size of the pertinent cell. The effects of this shear force largely depend on the properties of the cell itself. In comparison to microroganisms, insect cells and animal cells in general are very fragile. This is the result of their relatively large size and the lack of a cell wall (Chalmers, this book). In particular in larger bioreactors an adequate oxygen supply is hampered by this cell fragility. Special measures are thus required for fragile-cell bioreactors. Effective measures are addition to the medium of shear protectant such as pluronic (Papoutsakis *et al*., 1991), immobilization of the cells in macroporous supports (Martens *et al*., 1995) and optimization of the design and the sparging of the bioreactors. The latter is discussed in this chapter.

Strictly speaking, shear forces result from spatial differences in the levels of momentum across material stream lines in a moving body of fluid. In a (stirred) bioreactor, however, cells can encounter a variety of

other mechanical forces due to collisions with the vessel walls, the agitator, or other objects in the bioreactor. In addition, sparged gas bubbles subject the cell to surface tension forces and to fluid mechanical forces resulting from the motion, disengagement and bursting of bubbles, and from foaming. Here, all will be collectively referred to as shear and if possible quantified in terms of shear rate, shear stress, or smallest turbulent-eddy length. The relation between the shear stress τ ($N.m^{-2}$), the dynamic viscosity η ($N.s.m^{-2}$) and the shear rate $\dot{\gamma}$ (s^{-1}) is given by Newton's equation:

$$\tau = -\eta \frac{\partial v}{\partial x} = \eta \dot{\gamma} \qquad (1)$$

with *dv/dx* (s^{-1}) the fluid velocity gradient. The dynamic viscosity of real Newtonian fluids is a constant dependent only of pressure and temperature. Most biotechnological fluids are dilute aqueous media for which Newton's equation is appropriate. Furthermore, from a practical and engineering point of view, the existing relations for non-Newtonian behaviour, e.g. in case of very thick cell suspensions, are not very suitable for describing fluid flow in technical equipment. Therefore, only Newtonian fluids will be considered here.

Fluid flow can basically be divided into two types, i.e. laminar and turbulent flow. In laminar flow, fluid elements move along parallel stream lines. In this case shear stresses in the fluid are predictable from velocity gradients. This is not possible in turbulent flow. Hinze (1959) defines turbulent flow as follows: "Turbulent fluid motion is an irregular condition of flow in which the various quantities show a random variation with time and space coordinates, so that statisticlly distinct average values can be discerned". Turbulence can be generated by friction forces at solid objects, e.g. impellers, or by the flow of layers of fluid with different velocities part or over one another, for example as a result of air bubbles moving through the liquid. As turbulence is a common situation in bioreactors, much attention is paid to this aspect, moreover as especially turbulence can be lethal to fragile cells.

For the rational design and scale up of bioreactors the shear sensitivity of fragile cells should be described in quantitative terms. Experiments are usually the only way to collect quantitative data on the influence of shear on a particular cell type. The measured parameter for quantification of shear effects should be closely related to the aim of the technical process under investigation, for instance cell viability and growth, formation rate or quality of the desired product, yield coefficients, and overall productivity. Meijer (1989) expresses the opinion that shear-sensitivity data should preferably be collected in a down-scaled version of the intended production system. Therefore, if it is the intention to develop a large-scale process based on an impeller-stirred tank, the recommended method would be to collect data in a small impeller-stirred tank. Meijer recognizes the disadvantage of the poorly-defined and irregular shear levels in stirred vessels, but data collected in a device with well-defined and constant shear levels like viscometers, on the other hand, usually requires an awkward translation to the practical situation. Probably the best approach is to analyse the available shear-determining devices and damage-measuring methods for each particular cell type and process aim before making a choice. Here the focus is solely on stirred vessels, bubble columns and air-lift loop reactors, as these are in general the bioreactors of choice for larger-scale productions, also for fragile cells, despite of the relatively high level of shear. These well-mixed bioreactors have a variety of advantages such as (Kunas & Papoutsakis, 1990) scaleability, ease of controlling and monitoring important bioreactor parameters, relatively uniform bioreactor conditions, and use of existing industrial capacity and experience from other biological processes.

The stirred vessel

Introduction

The standard fermentor has been and still is the workhorse of the bioreactor stable used in biotechnology. Consequently, the practical experience with these stirred vessels is enormous and so is the desire to use them, also for fragile cells, despite the obvious disadvantage of poorly-defined, irregular and many times high shear levels. Many studies aiming at the determination of the fragility of cells have therefore been executed in stirred vessels (Van 't Riet & Tramper, 1991, and references cited therein). A detailed analysis of the hydrodynamic effects on anchorage-dependent animal cells attached to microcarriers in stirred vessels can be found in Cherry & Papoutsakis (1986), Croughan *et al.,* (1987, 1989), and Lakothia & Papoutsakis (1992). Animal cells on microcarriers are especially susceptible to sheear. In addition to the lack of a protective cell wall and their relatively large size (diameter of about 15 μm), they also lack individual cell mobility. Attached cells thus can not freely rotate or translate and

accordingly can not reduce the net forces and torques experienced in the shear fields of the moving fluids in a stirred vessel.

The purpose of stirring the medium in a bioreactor is threefold. Firstly, it is required to prevent the setting of the cells and secondly to assure a homogeneous environment for the cells, i.e. a continuous and adequate supply of nutrients. Thirdly, stirring is also used to improve the oxygen transfer from the gas to the liquid phase. In order to reach these goals, certainly the latter two, the stirrer speed usually needs to be so high that the fluid flow will be turbulent. Analysis of turbulent flow fields with respect to shear is therefore pivotal and will be discussed in some detail.

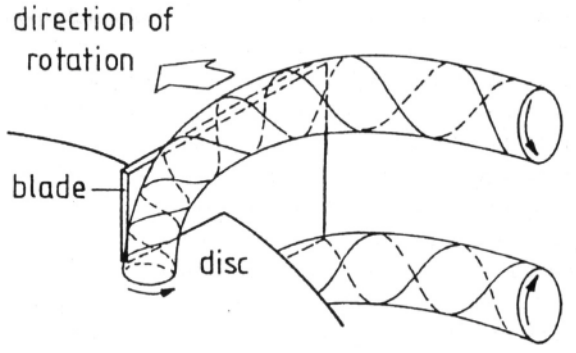

Figure 1. Schematic 3-dimensional view of the trailing-vortex pair in the stirrer blade region (adapted from Van 't Riet & Smith, 1975).

Smallest turbulent-eddy model

The effects of hydrodynamic forces on animal cells in a stirred bioreactor have been extensively studied in microcarrier systems, where the cells grow attached to the surface of spherical particles typically about 180 μm in diameter. The mechanisms that best explained the experimental results were interactions of the microcarrier beads with turbulent eddies having length scales smaller than the beads and collisions between the beads (Croughan *et al.*, 1987; Cherry & Papoutsakis, 1988; Croughan *et al.*, 1989). If these same explanations of damage mechanisms which work well for cells on microcarriers are applied to fragile cells in suspension, they fail (Cherry & Kwon, 1990). Individual cells of about 15 μm, which is a diameter typical for animal cells, are much smaller than the length scale of the smallest turbulent eddy in any reasonably agitated bioreactor (Croughan *et al.*, 1987; Cherry & Papoutsakis, 1988; Oh *et al.*, 1989) and therefore would be expected to be insensitive to damage from the eddy interactions that damage cells on microcarriers. Similarly, cells at a density of 10^{12} cells.m^{-3}, which is typical for animal-cell cultures when no perfusion is applied, occupy only about 0.1 vol % of the bioreactor and should have a negligible number of collisions (Beverloo & Tramper, 1994), especially since their density is so close to that of the medium that they will not deviate greatly from the fluid stream lines. However, also cells in suspension can be susceptible to excessive agitation, although the general level of sensitivity seems to be less than for anchored cells (Cherry & Kwon, 1990). In particular when the serum concentration is lowered in the culture medium, which is often practiced for economic and down-stream-processing reasons, the shear sensitivity increases and agitation can become rapidly too high unless other protective agents can be added. It is thus a situation to be reckoned with.

The common physical picture of turbulence starts with large eddies created in the case of a mechanically stirred vessel by an impeller (Figure 1). These large eddies pass their kinetic energy on to successively smaller eddies without loss until the energy is finally dissipated viscously as heat in eddies of some smallest size. In case of isotropic turbulence, which has no preferred direction, these smallest eddies have characteristic scales of length λ_K and velocity v_K (Kolmogorov theory):

$$\lambda_K = \left(\frac{v^3}{\varepsilon}\right)^{0.25} \tag{2}$$

$$v_K = (\varepsilon v)^{0.25} \tag{3}$$

with ε the empirical mass average of turbulent energy dissipation (m^2.s^{-3}) and v the kinematic viscosity (m^2.s^{-1}). The energy dissipation in a vessel is equal to the power consumption $P_s(W)$. The general equation for the power consumption is:

$$P_s = N_p \rho N^3 D^5 \tag{4}$$

with N_p the dimensionless stirrer power number, ρ the density of the liquid (kg.m^{-3}), N the stirrer speed (s^{-1}) and D the stirrer diameter (m). For fully turbulent conditions (Reynolds number = Re = $ND^2\rho/\eta \geq$ 10000) the dimensionless power number N_p for any stirrer type in a baffled vessel is constant (Van 't Riet & Tramper, 1991). For lower Re-numbers N_p is a function of Re only.

For insect cells in suspension, we have found that death rapidly occurs at a stirred speed N of about 9 s^{-1} in a 1 liter round-bottomed fermenter equipped with a marine impeller (diameter D = 4 cm). At this critical

stirrer speed Re calculated is 1440, thus far below fully turbulent conditions. From the appropriate graph in the above reference an N_p of about 1 can be read at this Re-number. Substituting this in Eq. 4 together with ρ = 1000 kg.m^{-3}, η = 0.01 N.s.m^{-2}, N = 9 s^{-1} and D = 0.04 m gives a P_s of 2.3 × 10^{-3} W, which in a stirred vessel of 1 dm^3 and a density of 1000 kgm^{-3} is equal to the mass average rate of energy dissipation (W.kg^{-1}). With a kinematic viscosity υ of 10^{-5} m^2.s^{-1} this yields for the mean Kolgomorov length scale (Eq. 2) λ_k = 1.44 mm, which is two orders of magnitude larger than an insect cell. The energy dissipation near the impeller is however much bigger. Oh *et al.* (1989) assume, as others have, that essentially all the energy is dissipated in half the volume occupied by the impeller. In that case, the Kolmogorov length scale in that region of the impeller is given by

$$\lambda'_K = \left(\frac{v^3}{\varepsilon'}\right)^{0.25} \tag{5}$$

where

$$\varepsilon' = 130\varepsilon \tag{6}$$

which gives a minimum Kolmogorov length scale (Eq. 5) of λ_K = 0.43 mm, still considerably larger than an insect cell.

Calculation of the mean maximum shear stress by means of the following equation derived by Cherry & Kwon (1990) for such a situation

$$\tau_{max} = 5.33\rho\sqrt{\varepsilon v} \tag{7}$$

yields 2.5 N.m^{-2}. Multiplying the mean energy dissipation first by a factor 130 (Eq. 6), yields for the maximum shear stress 29 N.m^{-2}. From studies with viscosimeters or other well-defined shear-stress devices, a shear stress of about 1 N.m^{-2} has been found to be the critical value for damage. From this, one could conclude that the maximum shear stress obtained from the mean energy dissipation is the more likely parameter for scale up than the one obtained from thee maximum energy dissipation in the impeller region. Other relations, also based on Kolmogorov's theory, yield shear stresses close to the critical value too and therefore earn further attention as scale-up parameters as well (Tramper *et al.*, 1993).

Implications for reactor design

As said above, there are 3 reasons for stirring a bioreactor. Firstly, it is required to prevent the settling of the cells. As a rule of thumb one can say that the minimum velocity in the bulk phase should be at least twice the terminal settling velocity of the cell. Stokes' law can be used to calculate the setting velocity v_s (m.s^{-1}) of a single cell as:

$$v_s = \frac{d_c^2(\rho_c - \rho)g}{18\eta} \tag{8}$$

in which ρ_c is the specific density of the cell (kg.m^{-3}). In order for Stokes' law to be valid, the cell Reynolds number, defined as

$$Re = \frac{\rho v_s d_c}{\eta} \tag{9}$$

should be less than 1.

In the intermediate range (1 < Re < 10^3) Beek & Mutzall (1975) give as relation for v_s:

$$C_w Re^2 = \frac{4d_c^3\rho(\rho_c - \rho)g}{3\eta^2} \tag{10}$$

in which c_w is the drag coefficient (–). The right hand side of Eq. (10) can be calculated for a given situation and by means of Figure 2 the relevant Re can be found, from which v_s can be calculated. Due to the very small difference in density of cell and medium, keeping cells in suspension generally requires very gentle stirring. For our insect-cell suspensions, assuming a density difference of 25 kg.m^{-3}, a v_s of 4.4 × 10^{-7} m.s^{-1} is calculated (Eq. 8) and with that a Re of 8 × 10^{-7} (Eq. 9), thus Eq. (8) is valid for this situation. Extreme low fluid velocities are thus required to keep the insect cells in suspension and this thus should not create problems from the point of view of shear sensitivity. Even cells attached to microcarriers generally need only very gentle stirring to keep them in suspension.

The primary reason for stirring cell-culture reactors is transfer of oxygen and maintaining homogeneity by minimizing variations throughout the reactor of dissolved oxygen and other nutrient concentrations or temperature. The average liquid velocity needed to give effective homogeneity can be estimated by requiring that the cell moves through the various areas of different conditions in an amount of time that is small compared to their biological respons time. In another chapter of this issue we have analyzed this aspect in detail. As the scale increases, liquid velocities, and thus turbulence, should be higher to ensure sufficient homogeneity. An analysis of shear and turbulence as given in this chapter is thus generally essential to be

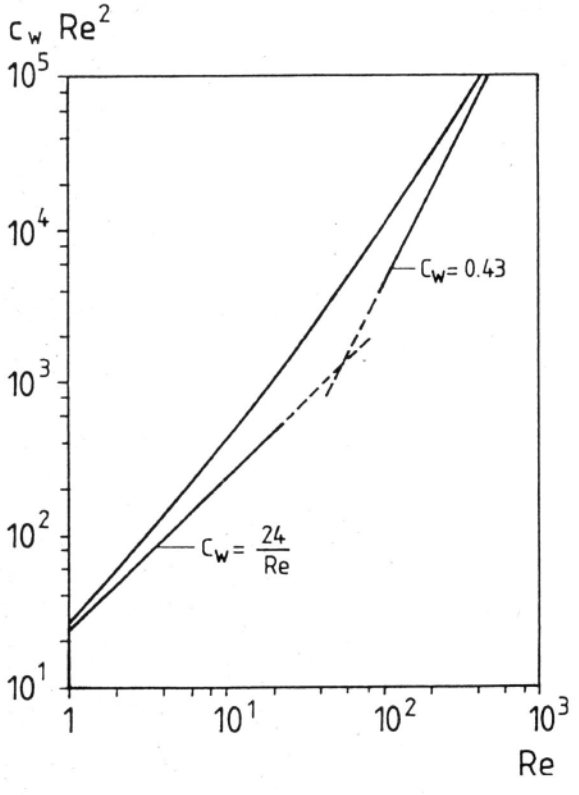

Figure 2. Drag coefficients and related functions fort spherical particles (adapted from Beek & Mutzall, 1975).

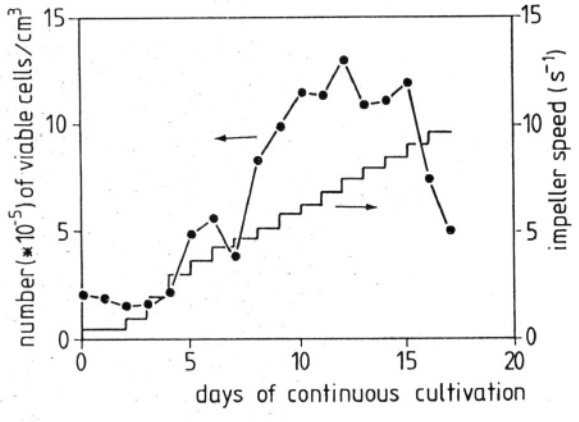

Figure 3. Insect-cell density in a continuous culture at increasing stirrer speed (adapted from Tramper *et al.*, 1986).

able to rationally design and scale up bioreactors meant for growth of fragile cells.

Stirring is also required for enhanced oxygen supply. Significant improvement can be accomplished by dispersion of the air bubbles in case of sparging, which is unavoidable if the size of the bioreactor increases. As a rule of thumb a tip speed of the impeller of about 2 $m.s^{-1}$ is needed. Figure 3 shows that insect cells die in a small stirred bioreactor (1.5 dm^3) if the impeller speed is larger than about 9 s^{-1}, which means a tip speed of 1.13 $m.s^{-1}$ (D = 0.04 m). At the time we found this we concluded that stirring from the point of substantially improving oxygen transfer by dispersion of air bubbles in insect-cell suspensions, but also for animal-cell suspensions in general, generally is impossible, unless shear protectants like pluronic can be added. Therefore we directed our research mainly to bubble-column and air-lift type of bioreactors. In the mean time it has been found that death of cells probably is the result of air bubbles drawn into the medium at this critical stirrer speed via the vortex (Kunas & Papoutsakis, 1990). This, however, does not change the principle of the analyses given in this chapter.

Bubble-column and air-lift bioreactors

Introduction

In contrast to the impeller-stirred vessels, bubble-column and air-lift type of bioreactors have been used relatively scarcely as devices to determine and quantify the fragility of cells, even though they are of great interest for use as bioreactors for growth of fragile cells (Katinger & Scheirer, 1982). In fact, only three or four groups have studied and used bubble columns and air lifts in this respect. Handa-Corrigan *et al.* (1989) particularly studied the effects of sparging on hybridomas and other mammalian cells in suspension culture. They found that damage of cells occurs especially during the bursting of the air bubbles at the suspension surface and that the nonionic surfactant pluronic has a concentration-dependent protective effect. The latter phenomenon has been studied extensively the last years and many recent papers describe and quantify this effect of pluronic and other polymers, both for insect cells (e.g. Goldblum, *et al.*, 1990) and animal cells in general (Papoutsakis, 1991).

Jordan *et al.* (1994) report that in the absence of surfactants, cells are killed if they come in contact with air bubbles. It surfactants are present in the medium the bubbles become saturated with these surfactants, which was shown by a decrease in rising speed of air bubbles in liquid without cells. In the case of partial saturation the cells adsorb to the bubbles without being killed, while fully saturated bubbles showed no interaction

with the cells. Adsorption of cells to bubbles has also been shown by Bavarian *et al.* (1991). Thus, according to the work of Jordan *et al.*(1994) events in the sparger region, where bubbles are not yet totally covered with surfactants, may contribute to cell death. Apart from this, Murhammer & Goochee (1990) also state that cell death in air-lift reactors may occur in the sparger region if a large pressure drop over the orifice is present.

At the bubble disengagement zone suspension cells present in the bubble film and near the cavity wall are killed as the bubble ruptures (Chalmers & Bavarian, 1991; Cherry & Hulle, 1992; Trinh *et al.,* 1994; Wu & Goosen, 1995). Trinh *et al.* (1994) calculated that a specific killing volume (see below) of 2×10^{-3} would correspond to a film thickness around the air-bubble of about 2 μm. Cherry & Hulle (1992) did the same calculation for a specific killing volume of 4×10^{-3} and found a value of 5 μm. According to Trinh *et al.* (1994) the thickness of 1 to 2 μm would imply that only cells attached to the bubble are killed. Cell attachment to the bubble is reported (Bavarian *et al.*, 1991; Jordan *et al.*, 1994) and will depend on the saturation of the bubble by surfactants (Jordan *et al.*, 1994). Extending this work, Chattopadhyay *et al.* (1995) showed that "additives that rapidly lower the liquid-vapor interfacial tension of the culture medium also prevent adhesion of cells to the bubble surface", thus lowering the specific killing volume. Whether or not this is the only interfacial phenomenon that explains the protective effect of additives remains to be investigated (Chattopadhyay *et al.*, 1995).

Wudtke & Schügerl (1987) investigated the fragility of insect cells using various methods, among others a bubble column. In agreement with the above-mentioned findings, these authors found that covering the suspension with a paraffin layer prevented the appearance of cell debris, indicating that the bubble bursting is indeed a damaging process. Quantitative relationships suitable for bioreactor design and scale up are not given. Good growth on a larger scale is however possible, both of insect cells (Maioeralla *et al.*, 1988) and animal cells in general (Birch & Arathoon, 1990).

Killing volume theory

To describe the death rate of suspension cells for the design of bubble-column and air-lift reactors the hypothetical killing volume model of Tramper *et al.* (van 't Riet & Tramper, 1991) may be used. The model assumes first-order death-rate kinetics and a hypothetical killing volume associated with each air bubble, in which all cells are killed. This results in the following equation for the first-order death-rate constant, $k_d(s^{-1})$:

$$k_d = \frac{24FX}{\pi^2 d_b^3 D^2 H} \tag{11}$$

where F is the gas flow rate ($m^3.s^{-1}$), d_b is the bubble diameter (m), D is the reactor diameter (m), H is the reactor height (m), and X is the hypothetical killing volume (m^3). Tramper *et al.* (1988) showed that the hypothetical killing volume was proportional to the bubble volume for bubble diameters in the range of 2–6 mm and thus Eq. 11 can be simplified to:

$$k_d = \frac{4FX'}{\pi D^2 H} \tag{12}$$

where X' is the specific hypothetical killing volume (–) being the hypothetical killing volume divided by the volume of the air bubble. Trinh *et al.* (1994) suggest on the basis of their results that this hypothetical killing volume is comprised of a very thin layer of fluid (1 to 2 μm) immediately surrounding the bubble cavity. Jordan *et al.* (1994) define the hypothetical killing volume as a real volume within which cells have a high probability of making contact with the bubble during the time that its surface is not fully saturated with surfactants molecules. Thus, according to these authors, the hypothetical killing volume represents a real volume associated with an air bubble.

Literature values for the specific hypothetical killing volume vary between 2×10^{-3} and 20×10^{-3}. This is caused by the fact that the specific hypothetical killing volume will depend on the cell line and type, serum concentration (Martens *et al.,* 1992; van der Pol *et al.*, 1990) and growth rate (Martens *et al.*, 1993), which are distinct in the experiments presented by the different authors. For insect cells in bubble columns Tramper *et al.* (1988) found a value of 4×10^{-3} at a serum concentration of 10%. For hybridoma cells in bubble columns Jöbses *et al.* (1991) reported a value of 7×10^{-3} (1% serum) and Van der Pol *et al.* (1990) a value of 9×10^{-3} (5% serum). For hybridoma cells in an air-lift reactor a value of 2×10^{-4} (5% serum) is found (Martens *et al.*, 1992). Because of the variations in experimental conditions, it is difficult to compare the results. What is clearly shown, however, is that the killing-volume theory applies to all these different cell lines and conditions, even to Vero cells on microcarriers (Martens *et al.*, 1995). Preliminary experimental validations in larger bubble-column and air-lift bioreactors indicate that also on pilot scale

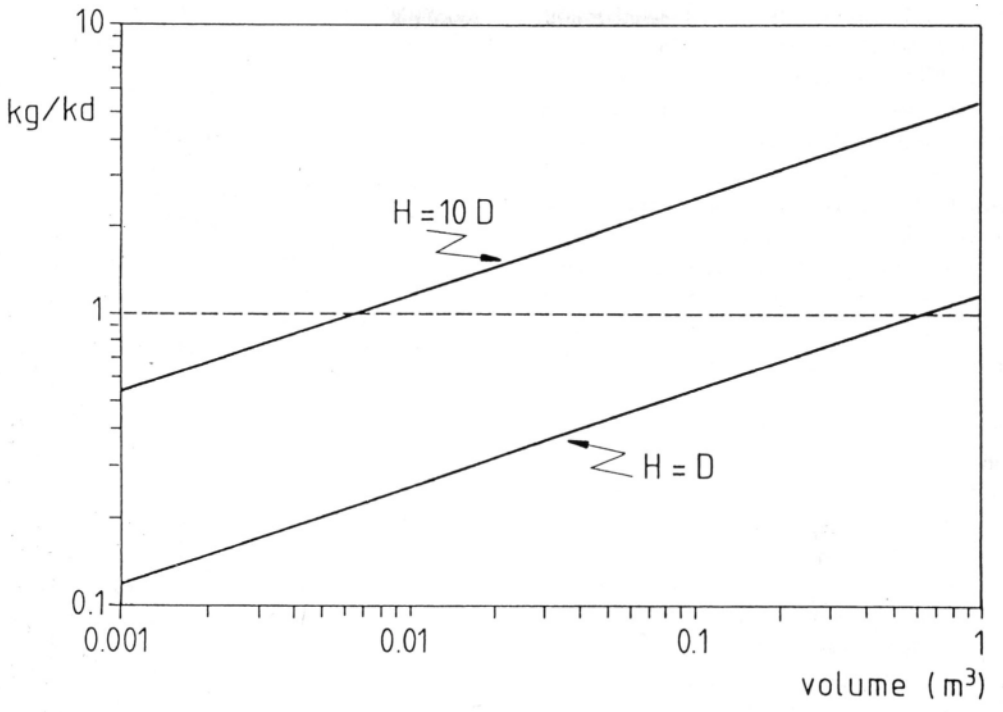

Figure 4. Ratio of the first-order growth and death rate constant as a function of the bubble-column working volume (height H parameter; from Tramper *et al.*, 1988).

the killing-volume model is applicable. The specific hypothetical killing volume is thus an easy and rapidly determinable fragility parameter which can be used for scale up as shown below.

Implications for reactor design

For scale up of fragile-cell cultures in which oxygen is supplied by sparging air through the suspension, it is important to correlate the (specific) hypothetical killing volume with the oxygen need of the cells. The specific oxygen transfer rate OTR ($\mathrm{mol.m^{-3}.s^{-1}}$) can be written as:

$$OTR = k_{ol}A(C_0^* - C_0) = OURC_x \qquad (13)$$

with k_{ol} the oxygen transfer coefficient ($\mathrm{m.s.^{-1}}$), A the specific surface area of the air bubbles ($\mathrm{m^2.m^{-3}}$), C_0^* the concentration of oxygen in the liquid when in equilibrium with air and C_0 the actual concentration ($\mathrm{mol.m^{-3}}$), and OUR the oxygen uptake rate of one unit of cells (mol oxygen per unit number, mol or kg cells). A can also be written as

$$A = \frac{n'\pi d_b^2}{0.25\pi T_v^2 H} \qquad (14)$$

with n' being the number of air bubbles present in the reactor:

$$n' = \frac{F_g h}{1/6\pi d_b^3 v_r} \qquad (15)$$

where v_r is the rising velocity of an air bubble relative to the vessel wall (m/s). Substitution in Eq. 14 gives:

$$A = \frac{24F_g}{\pi d_b v_r T_v^2}. \qquad (16)$$

From Eq. (13) the minimal specific surface area A_{min} is obtained:

$$OURC_x = k_{ol}A_{min}(C_0^* - C_{0,min}) \qquad (17)$$

with $C_{0,min}$ being the minimum liquid oxygen concentration at which cells are able to grow.

Growth of cells in a continuous culture can be described by first-order kinetics:

$$C_x(t) = C_x(0)e^{k_g t} \qquad (18)$$

with k_g the first-order growth-rate constant ($\mathrm{s^{-1}}$). In order for growth of cells to occur in a sparged reactor, k_d should be sufficiently smaller than k_g:

$$k_g >> k_d \qquad (19)$$

For designing a continuous culture of fragile cells in a bubble-column or air-lift reactor Eq. 12–19 can be used. Figure 4 shows the worked-out example for insect cells. Inspection of the equations for k_d and A reveals that especially the height H of the reactor and the oxygen tension in the gas C_0^* are the parameters to adjust in order to meet the demands set by the minimum specific surface area needed to supply sufficient oxygen and by the fact that the growth should be faster than the death rate. The effect of the height is clearly shown in Figure 4. The rising velocity v_r and the air-bubble diameter d_b are, in contrast, hardly adjustable parameters.

Conclusion

In this chapter we have attempted to evaluate the most important parameters which can be useful for the purpose of design and scale up. Insect cells and animal cells in general can be grown well in large vessels. However, none of the theories and parameters discussed in this chapter have been validated on a larger scale than laboratory and small pilot reactors. Selection of the most suitable design and scale-up method therefore needs in particular studies in larger vessels. The Kolmogorov theory and the killing-volume model are in this respect the most promising approaches for the optimal design of large-scale animal-cell bioreactors.

References

Bavarian F, Fan LS & Chalmers JJ (1991) Microscopic visualization of insect cell-bubble interactions I: rising bubbles, air-medium interfacee, and the foam layer. Biotechnol. Progress 7: 140–150.

Beek WJ & Mutzall KMK (1975) Transport Phenomena, Wiley, London.

Beverloo WA & Tramper J (1994) Intensity of microcarrier collisions in turbulent flow. Bioprocess Eng. 11: 177–184.

Birch JR & Arathoon R (1990) Large-scale cell culture in biotechnology. In: Large-Scale Mammalian Cell Culture Technology (Lubiniecki AS, ed.), Marcel Dekker, New Tork, 251.

Chalmers JJ & Bavarian F (1991) Microscopic visualization of insect cell-bubble interactions II: the bubble film and bubble rupture. Biotechnol. Progress 7: 151–158.

Chattopadhyay D, Rathman JF & Chalmers JJ (1995) The protective effect of specific medium additives with respect to bubble rupture. Biotechnol. Bioeng. 45: 473–480.

Cherry RS & Hulle CT (1992) Cell death in the thin films of bursting bubbles. Biotechnol. Prog. 8: 11–18.

Cherry RS & Papoutsakis ET (1986) Hydrodynamic effects on cells in agitated tissue culture reactors. Bioprocess Eng. 1: 29–41.

Cherry RS & Kwon K-Y (1990) Transient shear stresses on a suspension cell in turbulence. Biotechnol. Bioeng. 36: 563–571.

Cherry RS & Papoutsakis ET (1988) Physical mechanisms of cell damage in microcarrier cell culture bioreactors. Biotechnol. Bioeng. 32: 1001–1014.

Chisti Y & Moo-Young M (1989) On the calculation of shear rate and apparent viscosity in airlift and bubble column bioreactors. Biotechnol. Bioeng. 34: 1391–1392.

Croughan MS, Sayre ES & Wang DIG (1989) Viscous reduction of turbulent damage in animal cell cultures. Biotechnol. Bioeng. 33: 862–872.

Croughan MS, Hamel JF & Wang DIC (1987) Hydrodynamic effects on animal cells grown in microcarrier cultures. Biotechnol. Bioeng. 29: 130–141.

Goldblum S, Bae Y-K, Hink WF & Chalmers JJ (1990) Protective effect of methylcellulose and other polymers on insect cells subjected to laminar shear stress. Biotechnol. Prog. 6: 383–390.

Handa-Corrigan A, Emery AN & Spier RE (1989) Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles. Enzyme Microb. Technol. 11:230–235.

Henzler H-J & Kauling DJ (1993) Oxygenetation of cell cultures. Bioprocess Eng. 9: 61–75.

Hinzer JO (1959) Turbulence, McGraw-Hill, New York.

Jöbses I, Martens DE & Tramper J (1991) Lethal events during gas sparging in animal cell culture. Biotechnol. Bioeng. 37: 484–490.

Jordan M, Sucker H, Einsele A, Widmer F & Eppenberger HM (1994) Interactions between animal cells and gas bubbles: the influence of serum and pluronic F68 on the physical properties of the bubble surface. Biotechnol. Bioeng. 43: 446–454.

Katinger HWD & Scheirer W (1982) Status and development of animal cell technology using suspension culture techniques. Acta Biotechnologica 2: 3–41.

Kunas KT & Papoutsakis ET (1990) Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment. Biotechnol. Bioeng. 36: 476–483.

Lakothia S, Bauer KD & Papoutsakis ET (1992) Damaging agitation intensities increase DNA synthesis rate and alter cell-cycle phase distributions of CHO cells. Biotechnol. Bioeng. 40: 978–990.

Maiorella B, Inlow D & Harano D (1988) USA Patent, PCT/US88/02444.

Martens DE, De Gooijer CD, Beuevery EC & Tramper J (1992) Effect of serum concentration on hybridoma viable cell density and production of monoclonal antibodies in CSTR's and on shear sensitivity in air-lift loopreactors. Biotechnol. Bioeng. 39: 891–897.

Martens DE, De Gooijer CD, Van der Velden-de Groot CAM, Beuvery EC & Tramper J. (1993) Effect of dilution rate on growth, productivity, cell cycle and size, and shear sensitivity of a hybridoma cell in a continuous culture. Biotechnol. Bioeng. 41: 429–439.

Martens DE, Nollen EAA, Hardeveld M, Van der Velden-de Groot CAM, De Gooijer CD, Beuvery EC & Tramper J Death rate in a small air-lift loop reactor of vero cells grown on solid microcarriers and in macroporous microcarriers. Submitted.

Meijer JJ (1989) Effects of hydrodynamic and chemical/osmotic streess on plant cells in a stirred bioreactor, Ph.D. thesis, Technical University Delft.

Murhammer DW & Goochee CF (1990) Structural features of nonionic polyglycol polymer molecules responsible for the protective effect in sparged animal cell bioreactors. Biotechnol. Prog. 6: 142–148.

Murhammer DW & Goochee (1990) Sparged animal cell bioreactors: mechanism of cell damage and pluronic F-68 protection. Biotechnol. Prog. 6: 391–3197.

Oh SKW, Nienow AW, Al-Rubeai M & *Emery* AN (1989) The effects of agitation intensity with and without continuous sparging on the growth and antibody production of hybridoma cells. J. Biotechnol. 12: 45–62.

Papoutsakis ET (1991) Media additives for protecting animal cells against agitation and aeration damage in bioreactors. Trends in Biotechnol. 9: 316–324.

Tramper J, de Gooijer CD & Vlak JM (1993) Scale-up considerations and bioreactor development for animal cell cultivation. In: Insect cell culture engineering (Goossen MFA, Daugulis AJ & Faulkner P, eds.), Marcel Dekker, New York, 139–177.

Tramper J, Smit D, Straatman J & Vlak JM (1988) Bubble-column design for growth of fragile insect cells. Bioprocess Eng. 3: 37–41.

Tramper J, Williams JB, Joustra D & Vlak JM (1986) Shear sensitivity of insect cells in suspension. Enzyme Microb. Technol. 8: 33–36.

Trinh K, Garcia-Briones M, Hink F & Chalmers JJ (1994) Quantification of damage to suspended insect cells as a result of bubble rupture. Biotechnol. Bioeng. 43: 37–45.

Van 't Riet K & Smith JM (1975) The trailing vortex system produced by Rushton turbine agitators. Chem. Eng. Sci. 30: 1093–1105.

Van 't Riet K & Tramper J (1991) Basic Bioreactor Design, Marcel Dekker, New York.

Van der Pol L, Zijlstra G, Thalen M & Tramper J (1990) Effect of serum concentration on production of monoclonal antibodies and on shear sensitivity of a hybridoma. Bioprocess Eng. 5: 241–245.

Wu J & Goosen MFA (1995) Evaluation of the killing volume of gas bubbles in sparged animal cell culture bioreactors. Enzyme Microb. Technol. 17: 241–247.

Wudtke M & Schügerl K (1987) Investigation of the influence of physical environment on the cultivation of animal cells. In: Rheologie und mechanische Beanspruching biologischer Systeme, GVC.VDI, Düsseldorf, 159–173.

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Oxygen gradients in small and big sparged insect-cell bioreactors

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Introduction

Gradients are known to exist in fermenters. In particular during microbial fermentations on a large scale, especially oxygen gradients can be very steep, due to the low solubility of oxygen in aqueous media. Even though the respiration rates of animal cells are relatively low, oxygen gradients are also likely to occur in animal-cell bioreactors. It is unknown, however, whether this is only deleterious to the cells, or that also positive effects can be expected under certain circumstances. In this chapter the extent of oxygen gradients which can be expected in various bioreactors is estimated.

The stirred vessel is the workhorse in biotechnological fermentations; this is not different for animal-cell cultivations. In the latter field $10\ m^3$ is about the largest size in use and therefore this size is taken as the example to calculate gradients in. As a reference a 0.01 m^3 vessel is taken, which is approximately the largest bench-scale bioreactor. In addition to stirred vessels, air-lift loop reactors are used on these scales to a limited extent; evaluation of gradients in these loop bioreactors is therefore included as well. Bubble columns are not used much beyond the scale of $0.01\ m^3$ in the practice of animal-cell technology, but in this case study, for the sake of comparison, estimations of gradients in this type of bioreactor are done on the $10\ m^3$ scale too.

Gradients

Theory

Figure 1 illustrates the process of oxygen transfer by i) molecular diffusion from gas bubbles to the bulk-liquid phase, ii) mixing in the latter phase by convection, and iii) again molecular diffusion from the bulk-liquid phase to the surface of spherical particles through the surrounding stagnant layer. A particle in this study is either the cell itself, a microcarrier with cells attached to the surface, or a macroporous support with cells immobilized inside. In the latter case also molecular diffusion in the porous support is involved, as well as in micro-colonies of cells if these exist (Wijffels *et al.*, 1995). Assuming that, as result of heterogeneous cell growth due the diffusion limitation, eventually most of the cells are present as a thin layer just underneath the surface of the macroporous particles, an estimation of the number of cells per particle is obtained from a mass balance incorporating oxygen diffusion and consumption.

The equation describing molecular diffusion through the stagnant layer of oxygen-consuming particles reads for the steady state:

$$OTR' = K_l.A.\Delta C = k_l.A.(C_{\text{bulk}} - C_{\text{surface}}) = \\ = OUR' \qquad (1)$$

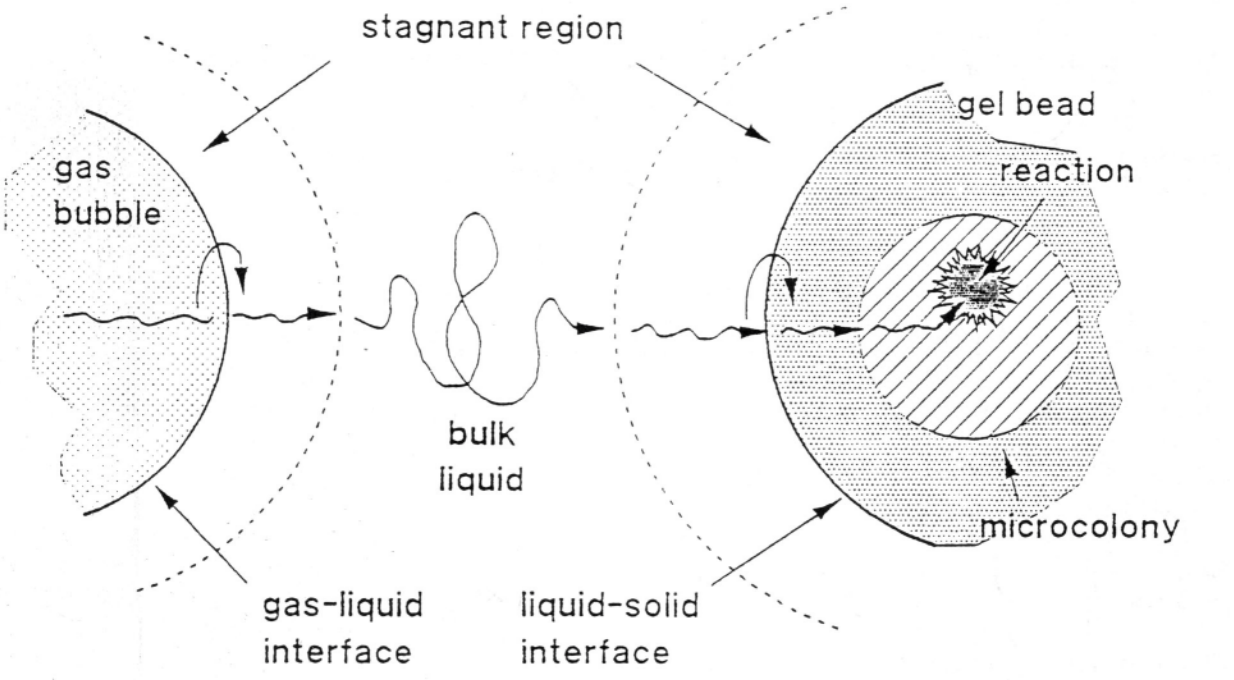

Figure 1. Schematic representation of the steps involved in transport of oxygen from a gas bubble to inside a cell (adapted from Bailey & Ollis, 1986).

with *OTR´* ($mol.particle^{-1}.s^{-1}$) the oxygen transfer rate through the stagnant layer of one particle, k_l ($m.s^{-1}$) the oxygen transfer coefficient, A ($m^2.particle^{-1}$) the surface area of a particle, ΔC ($mol.m^{-3}$) the oxygen concentration gradient over the stagnant layer, C_{bulk} and $C_{surface}$ ($mol.m^{-3}$) the oxygen concentration in the bulk liquid and at the surface of the particle, respectively, and *OUR'* ($mol.particle^{-1}.s^{-1}$) the oxygen consumption rate per particle, which in the steady state is thus equal to the oxygen transfer rate. To calculate ΔC, the diameter of the spherical particle (to determine A), the oxygen transfer coefficient, the number of cells per particle and the oxygen consumption rate per cell are thus required. For the latter the convenient value of 10^{-16} $mol.cell^{-1}.s^{-1}$ is taken, which is about the average value found in our laboratory for insect cells (*Spodoptera frugiperda*) and close to the value found by Kamen *et al.* (1991), and at the middle/high side of the values found in literature for other animal cells (e.g. Spier & Griffiths, 1984; Aunins & Henzler, 1993). This value multiplied by the number of cells per particle gives the particle oxygen consumption rate, assuming that all cells consume oxygen at the same rate, which can be seen as a worst-case scenario in this study. Oxygen transfer coefficients are estimated from Sherwood-type of relationships (Van't Riet & Tramper, 1991). For bubble columns and air lifts this estimation is done at two air-flow rates: rather low and average. High air flows are not considered, because it is unlikely that they can be used in animal-cell cultures since they cause high shear and foaming.

The bulk-liquid phase circulates through the bioreactors. In general, aeration occurs only locally and therefore oxygen may be exhausted in the non-aerated parts. Comparison of the times spent in the latter parts with the exhaust times calculated for three cell densities (a common one, a rather high one, and a desirable high one) gives an idea of the gradients which can exist in the bulk-liquid phase. Liquid flow velocities and empirical correlations for circulation times are used in these estimations. Gradients are also likely to occur in the liquid stagnant layer surrounding the air bubbles, contrary to the gas stagnant layer in the air bubble, due to high partition coefficient and the high diffusivities in gas. Air-bubble size and hold up are not very well defined and thus the calculation of the specific interfacial area a ($m^2.m^{-3}$) is not very accurate. Therefore empirical correlations for the product of the

oxygen transfer coefficient k_l and specific interfacial area a have been used here in the estimations of the gradients in the stagnant liquid layer surrounding the gas bubbles. The surface area A of one particle in equation (1) is in this case replaced by a, and OTR' and OUR' by OTR and OUR, respectively, the volumetric oxygen transfer and consumption rate ($mol.m^{-3}.s^{-1}$).

Activity of particles

To estimate the gradient in the stagnant layer surrounding particles, the oxygen consumption rate of the particles OUR' ($mol.particle^{-1}.s^{-1}$) must be known. In case of freely suspended cells the value is assumed to be 10^{-16} $mol.s.^{-1}.cell^{-1}$ (see above). The number of cells on a microcarrier is roughly estimated as follows. The surface area of a microcarrier with a diameter d_p of 185 μm is $\pi d_p^2 = 1.1 \times 10^{-7}$ m^2. It is assumed that, as result of spreading, the "cross-sectional" area occupied by one cell of diameter $d_C = 15$ μm is d_c^2, i.e. 2.25×10^{-10} m^2. At complete coverage (confluency) about 470 cells can thus be attached, which is a rather high value, since values as low as 150 and 70 cells per microcarrier have been found (N. Kalogerakis, personal communication). In the calculations here an average value of 300 cells per microcarrier has therefore been used.

To calculate the number of cells in the macroporous support particles, it is assumed that in systems with actively growing cells eventually a thin but dense layer of cells is formed just underneath the surface as result of intra-paticle diffusion limitation (Wijffels *et al.*, 1991). Compared to the radii of the macroporous supports considered here, i.e. 1.25 and 6 mm, this layer is relatively small (see below) and is therefore for the calculations considered to be a flat layer. The oxygen concentration profile in this layer of thickness δ, assuming a uniform oxygen consumption rate r ($mol.m^{-3}.s^{-1}$) throughout this layer of cells, can be described by (derived from a mass balance):

$$C(x) = C(0) + r.x^2/(2D_e)(0 \leq x \leq \delta, \quad (2)$$

with C(x) the concentration ($mol.m^{-3}$) at a distance x (m) from the side where the concentration reaches a minimum, C(0) ($mol.m^{-3}$). This value is assumed to be 0 $mol.m^{-3}$ (*Spodoptera frugiperda* cells grow well in continuous culture at almost zero oxygen concentration). D_e is the diffusion coefficient in the porous support with cells and assumed here to be 10^{-9} $m^2.s^{-1}$. This value is about 40% (see below) of the one in water, and roughly an average of the many which can be found in literature for immobilized-cell systems. If we assume that on average the space taken by one cell, including associated support and surrounding fluid, is d_c^3, than $r = 10^{-16} / (15 \times 10^{-6})^3 = 0.03$ $mol.m^{-3}.s^{-1}$. For most macroporous supports this means that about 60% of the volume is taken by solid material (cells plus support material) and 40% by liquid. This is the reason why the above value for the diffusion coefficient is chosen. When it is further assumed that gradients outside the particle can be neglected, i.e. the surface concentration corresponds to air saturation or $C(\delta) = 0.24$ $mol.m^{-3}$, then the thickness of the layer can be calculated by substituting the numerical values and $x = \delta$ in equation (2). This gives $\delta = 0.127$ mm (i.e. one tenth of the diameter of the small porous support, so the assumption of a flat layer was reasonable), which corresponds to about 8 cell layers. Even though the cells will respire less at the lower oxygen concentration in the deeper layers, it probably still is a good estimate as $C(\delta)$ always will be less than air saturation. Eight cell layers is also the number Palsson reported at the 1994 ESACT/JAACT-meeting in Veldhoven (NL) for hematopoietic cells in a membrane reactor in which, similarly, oxygen supply is by diffusion only. The volume taken by the cell layer in a porous support with $d_p = 1.25$ mm is $\pi.d_p^2.\delta = 6.2 \times 10^{-10}$ m^3, which corresponds to 1.84×10^5 cells. For a porous support with $d_p = 6$ mm this is 1.44×10^{-8} m^3 and 4.27×10^6 cells.

Gradients near particles

In addition to the particle oxygen consumption rate, also the liquid/solid oxygen transfer coefficient k_l of the particles must be known to calculate the gradient ΔC (equation 1) in the stagnant layer surrounding the particles. Due to its nature the surface of a solid particle can usually be considered as rigid. The k_l equations are therefore of the Sherwood type (Van 't Riet & Tramper, 1991). The k_l value is dependent on the velocity of the particle relative to the liquid. This velocity usually is unknown, but depends among others on particle diameter and the density difference between particle and liquid, which typically has a value of about 25 and 50 $kg.m^{-3}$ for the difference with cells and supports, respectively. In Van 't Riet & Tramper (1991) a procedure has been proposed which has been used to estimate this velocity and from that the k_l values in stirred vessels. Results thus obtained vary between 3×10^{-4} $m.s^{-1}$ for an individual cell and 4×10^{-5} $m.s^{-1}$ for the macroporous-support particles of 6 mm.

Table 1. Gradients in the stagnant layers surrounding particles

d_p (m)	OUR (mol.particle^{-1}.s^{-1})	k_l (m.s^{-1})	δ (μm)	ΔC (Mol.m^{-3})
15×10^{-6}	10^{-16}	3×10^{-4}	8	5×10^{-4}
200×10^{-6}	3×10^{-14}	4×10^{-4}	6	6×10^{-4}
1.25×10^{-3}	1.85×10^{-11}	5×10^{-5}	48	4×10^{-2}
6×10^{-3}	4.25×10^{-10}	4×10^{-5}	60	0.25

For calculations of k_l in bubble columns the relation of Sano *et al.* (1974) is used. Values found in this case lie between 1.2×10^{-5} m.s^{-1} for the largest macroporous support particles at the rather low air superficial velocity (10^{-3} m.s^{-1}) and 4.5×10^{-4} m.s^{-1} for an individual cell at the average air superficial velocity (10^{-2} m.s^{-1}).

For the calculations of the air-lift loop bioreactors the relation of Sänger & Deckwer (1981) is used. The values are comparable to those of the bubble columns.

Now having estimated the particle oxygen consumption rates and the oxygen transfer coefficients, the gradients around the various particles can be calculated from equation (1). Table 1 gives a summary of the results for some representative k_l values. It should be noted that a gradient here is the concentration difference over the thickness δ of the stagnant layer. These δ's have been calculated from $\delta = D/k_l$, in which D is the oxygen diffusion coefficient in the aqueous medium, i.e. 2.4×10^{-9} m^2.s^{-1}. The values in Table 1 show that the stagnant layer increases when the diameter increases for particles of the same density.

It is clear from Table 1 that only in case of the macroporous supports significant gradients can be expected in the stagnant layer. For the 1.25 mm particle the use of air to supply the oxygen does not necessarily create problems for the situations assumed, but it obviously does for the 6 mm particle, because at air saturation of the bulk-liquid phase the maximum possible gradient is 0.24 mol.m^{-3}, while 0.25 mol.m^{-3} is needed. Consequently, cell growth will be limited by external diffusion, resulting in a thinner cell layer than calculated above.

Gradients in the bulk phases

In addition to gradients in the stagnant layer surrounding the particles, gradients in the bulk-liquid phase of the various bioreactors may occur as well. The oxygen gradient will largely be determined by the time fluid elements spend in the unaerated zone of the pertinent bioreactor and by the volumetric oxygen consumption rate. The latter depends on the cell density. For the calculations a variable cell density of 10^{12}, 10^{13} and 10^{14} cells.m^{-3} is chosen. The first value is at the low side and typical for continuous cultures with suspended cells. The middle value is rather high, but can be approached in some batch cultures and realized in perfusion cultures with microcarriers and macroporous carriers. The last value certainly is high and strived for. Note that both the last two values can not be reached with the largest carrier size considered here. The time fluid elements spend in the unaerated zone depends among others on the type of bioreactor considered. Therefore the various bioreactor types will be discussed sequentially now.

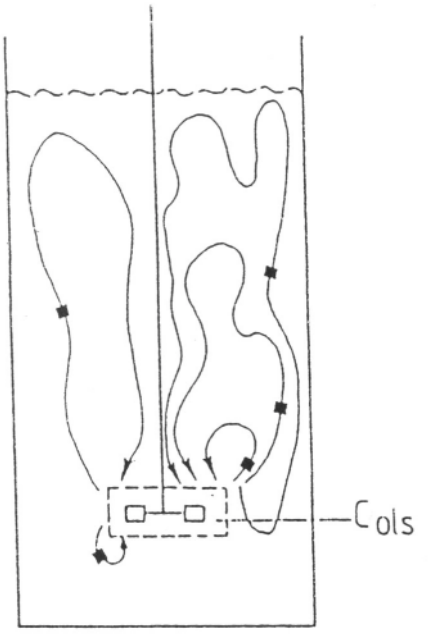

Figure 2. Schematic representation of trajectories of (segregated) fluid elements (■) and region where oxygen (C_{ols}) mass transfer occurs in a stirred vessel (from Van 't Riet & Tramper, 1991).

The stirred vessel

The fluid circulation patterns in a stirred vessel are shown in Figure 2. Circulation time rather than mixing time is a good measure for the time the fluid elements spend on average in the unaerated zone of the vessel. This is only true for situations that the stirrer speed is fast enough to disperse the air bubbles, i.e. the tip speed of the stirrer is at least 2 m.s^{-1}. In that case oxygen supply is very efficient but merely occurs in a small

Table 2. Circulation times in stirred tanks

	V (m^3)	N (s^{-1})	t_c (s)
Re = 1000	0.01	0.26	140
	10	2.6×10^{-3}	2×10^4
Re = 10,000	0.01	10	6
	10	1	59

area around the stirrer. If no dispersion of air bubbles takes places, thus at stirrer speeds below $2\ m.s^{-1}$, the overall oxygen supply is less efficient, but it occurs in a larger part of the reactor. An empirical correlation which can be used for estimation of the circulation time t_c in a stirred vessel is:

$$t_c = \frac{1.5}{N} \frac{(T_v' D)^3}{(N_p)^{1/3}} \frac{H_v}{T_v} \qquad (3)$$

with N the stirrer speed (s^{-1}), T_v and H_v the diameter and height, respectively, of the vessel (m), D the stirrer diameter (m), and N_P the power number, which is a dimensionless constant, but in fact only constant at a high Reynolds number ($Re > 10{,}000$), i.e. at fully turbulent conditions. Equation (3) is valid for aerated vessels with one stirrer only; increasing the number of stirrers proportionally reduces the ratio H_v/T_v. For re $< 10{,}000$ few data are available for large-scale bioreactors. Between $1000 < Re < 10{,}000$ the influence on $N.t_c$ is however limited. Therefore, rough predictions can be made using equations (3). The presence of rising air bubbles might improve the mixing to some extent in this regime, but for large-scale applications it is unknown to what extent. The situation below Re $= 1000$ should be prevented, as circulation and mixing times exponentially increase to unacceptable levels. Table 2 gives the results for the two considered vessel sizes at two Re numbers, which can be considered the extremes in animal-cell technology. The lower value for reasons stated above; the higher one to accomplish dispersion of air bubbles at the lowest shear level (i.e. a tip speed of $2\ m.s^{-1}$), which in many cases may even be too high for most animal cells due to their fragility, in particular when no shear protectants such as Pluronic-F68 can be used.

Table 2 clearly shows that the calculated circulation time in the large vessel at low Re is unacceptably large and unrealistic; the rising air bubbles obviously will have an improving effect in this case.

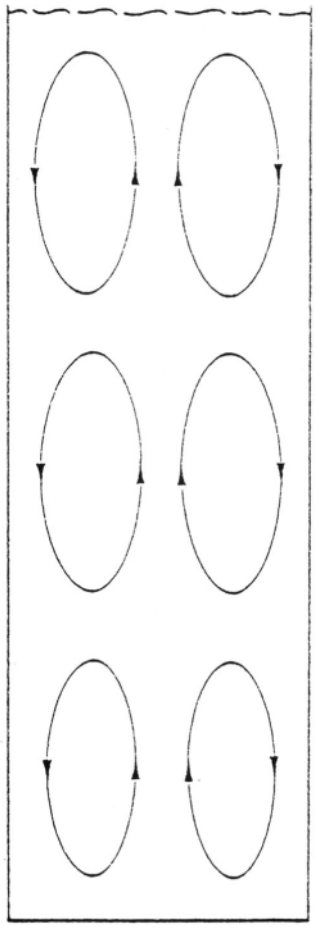

Figure 3. Schematic representation of the time-averaged circulatory flows in a bubble column (from Van 't Riet & Tramper, 1991).

The bubble column

The situation for a bubble column is quite different. Bubbles that originate at the sparger will rise in the column as a bubble swarm. If there are no circulatory flows, the flow is defined as homogeneous. In that case all the bubbles will rise with the same upward velocity. Mixing originates only from the entrained liquid in the wake of each bubble. This mixing effect, however, is limited. Homogeneous flow can only occur when the sparger holes are evenly distributed on the bottom of the vessel. Even in that case it occurs only at low superficial gas velocities. It is however a situation which in animal-cell technology has to be reckoned with, because aeration will usually be limited to minimize cell damage.

Table 3. Circulation times in bubble columns

V (m^3)	T_v (m)	H_v (m)	v_{gs} ($m.s^{-1}$)	t_c (s)
10^{-3}	1.85×10^{-1}	3.7×10^{-1}	10^{-3}	4.2
	1.85×10^{-1}	3.7×10^{-1}	10^{-2}	1.9
	1.1×10^{-1}	1.08	10^{-3}	14.6
	1.1×10^{-1}	1.08	10^{-2}	6.8
10	1.84	3.7	10^{-3}	19.1
	1.84	3.7	10^{-2}	8.9
	1.08	**11**	10^{-3}	67
	1.08	**11**	10^{-2}	31

If the sparger holes are not evenly distributed or if the gas flow is high, local differences in liquid velocities will occur. This leads to differences in hold-up and to differences in hold-up distribution in the bubble column. In that case a stabilized flow regime exists, in which an upward current occurs at the center of the column, where oxygen transfer predominantly happens, and a downward current at the outer side of the column. In tall columns the circulation is divided into loops that have a height of about the column diameter. This so-called heterogeneous flow regime is given schematically in Figure 3. Nearly all commercial bubble columns show the heterogeneous flow regime. For such bioreactors the circulation time t_c can be described as:

$$t_c = \frac{2.8}{\sqrt[3]{(g.v_{gs}/T_v^2)}} \qquad (4)$$

with g the gravitational acceleration ($m.s^{-2}$) and v_{gs} the gas superficial velocity ($m.s^{-1}$). This t_c is a rough estimate of the time fluid elements spend in the unaerated part of the bubble column. The results of the calculations using this equation are given in Table 3.

In addition to two gas superficial velocities and two sizes, two geometries are considered as well, i.e. a shallow and wide column, and a tall and slender one. Bold prints in the table are given to indicate that the column heights exceed 10 meters. In that case exhaustion of the air bubbles can start to play a significant role near the top of the column.

The air-lift loop reactor

For air lifts (Figure 4) the situation again is different from bubble columns and stirred vessels. The driving force for the liquid circulation is the density difference between riser and downcomer. For animal-cell cultures the air flow will usually be rather low to minimize cell damage and foaming. It is therefore a reasonable assumption that in the downcomer no air bubbles are present and that aeration only occurs in the riser. The unaerated part of the bioreactor is thus the downcomer. From the liquid velocity v_{lds} ($m.s^{-1}$) in the downcomer the time the fluid elements spend there can be calculated. However, this velocity can only be calculated from the superficial gas velocity v_{gs} iteratively (Verlaan, 1987). Table 4 gives the results of these calculations for two air-lift sizes, two diameter/height ratios, two superficial gas velocities, and a downcomer diameter T_d which is half the riser diameter T_r.

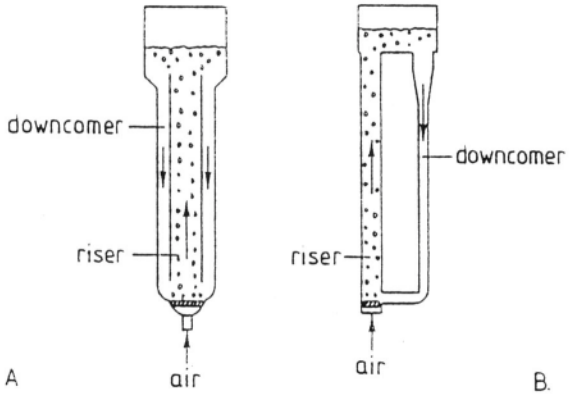

Figure 4. Schematic representation of the air-lift loop reactor. A: Internal loop. B: External loop (from Van 't Riet & Tramper, 1991).

Table 4. Times spend in downcomer of air lifts ($T_d = 0.5\ T_r$)

V (m^3)	T_r (m)	H_v (m)	v_{gs} ($m.s^{-1}$)	v_{lds} ($m.s^{-1}$)	t_d (s)
10^{-2}	0.1	1	10^{-3}	0.2	5
			10^{-2}	0.54	1.8
	0.17	0.34	10^{-3}	0.14	2.4
			10^{-2}	0.39	0.9
10	1	**10**	10^{-3}	0.48	21
			10^{-2}	1.21	8.3
	1.71	3.4	10^{-3}	0.37	9.1
			10^{-2}	0.96	3.6

Gradients in the bulk-liquid phase

In order now to be able to estimate if gradients will exist in the various bulk-liquid phases, the exhaust times of unaerated fluid elements must be known first. It is assumed above that the oxygen respiration rate of a cell is 10^{-16} $mol.cell^{-1}.s^{-1}$. For a rather low cell

density of 10^{12} cells.m^{-3}, which is typical for continuous cultures, the volumetric oxygen consumption rate is 10^{-4} mol.m^{-3}.s^{-1}. Knowing that the oxygen concentration of air-saturated liquid is 0.24 mol.m^{-3}, the exhaust time is 2400 s or 40 minutes. Comparing this figure with the above calculated circulation and liquid retention times, it is clear that only in the larger stirred vessel at the lowest Re number, a gradient may exist, though unlikely as a result of the mixing by rising air bubbles. Similarly, it can be calculated that in a suspension with the rather high cell density of 10^{13} cells.m^{-3} the exhaust time is 240 s or 4 minutes. From an inspection of Table 2 it is obvious that in all cases of the stirred vessel significant gradients exist and that in one case even complete exhaustion very likely will occur. The situation for the bubble column is considerably better (Table 3). Only in the larger, slender bubble column at the lower gas flow rate a considerable (up to 25%) decrease in oxygen concentration is likely. The gradients in the air lifts are mostly small (Table 4).

For the desirably high cell density of 10^{14} cells.m^{-3}, the exhaust time is 24 s. It is clear that in most cases considered steep gradients will exist, even in air lifts, and that in many cases complete exhaustion will occur rapidly and remain for quite long times.

If we consider tissue as well, having a cell density of about 10^{15} cells.m^{-3}, the exhaust time calculated in the same manner is 2.4 seconds. Although this is not more than a very rough estimate, it makes one thing overly clear and that is that continuous oxygen supply over a short distance is essential for tissue.

Gas/liquid transfer

So far, gradients in the stagnant liquid layer surrounding air bubbles have been neglected. However, in many cases these will be considerable, making the chances for complete exhaustion even higher. As an example, an empirical correlation is used to make rather rough estimations of the $k_l a$ values in bubble columns. Only actual measurements can yield more reliable results. The equation used for bubble columns is:

$$k_l a = 0.32(v_{gs})^{0.7} \qquad (5)$$

which applies to coalescing, non-viscous liquids at a temperature of 20 °C, with the annotation that the accuracy is not greater than ± 30%. The results of using this equation for the two superficial gas velocities and the three cell densities are given in Table 5.

It is clear that at higher cell densities, i.e. at higher oxygen uptake rates, very steep oxygen gradients exist

Table 5. Gas/liquid O_2-transfer in bubble columns

V_{gs} (m-s^{-1})	$k_l a$ (s^{-1})	OUR (mol.m^{-3}.s^{-1})	ΔC (mol.m^{-3})
10^{-3}	2.5×10^{-3}	10^{-4}	0.04
		10^{-3}	**0.39**
		10^{-2}	**3.9**
10^{-2}	1.3×10^{-2}	10^{-4}	$8\ 10^{-3}$
		10^{-3}	0.08
		10^{-2}	**0.79**

(marked bold in Table 5), in particular at the lower gas flow rates. Even the use of pure oxygen is not sufficient for all cases; also higher gas flows to increase the $k_l a$ are needed to improve the situation.

At equal power input the stirred vessel (power input: stirring) and the air lift (power input: gas compression) perform at best equal to the bubble column (power input: gas compression) from the point of view oxygen transfer. Although gross empirical correlations exist for stirred vessels (Van 't Riet & Tramper, 1991), it would lead too far here to work these out. It suffices to state that the situation is even more severe for both the stirred vessel and the air lift than for the bubble column. High stirrer speeds and high air flows are needed to substantially increase the $k_l a$ values.

Conclusions

It should be clear from the above that the calculations described here are at best rough estimations yielding order-of-magnitude values. Even though, the following general conclusions can be drawn. The gradients in stagnant layers surrounding the particles which are characteristic for animal-cell bioreactors are relatively small as compared to the gradients which can be expected in the bulk-liquid phases of the three bioreactors considered, in particular to the gradients in the stagnant layer surrounding the air bubbles. It can be concluded that under almost all circumstances gradients are likely to exist and can be very steep in larger vessels and in particular at high cell densities. The effects of gradients, however, are largely unknown; therefore research on the effects of gradients on specific and volumetric productivities and product quality seems to be an interesting area.

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References

Aunins JG & Henzler H-J (1993) Aeration in cell culture bioreactors. In: Stephanopoulos G (ed) Biotechnology Vol. 3. Bioprocessing, VCH, Weinheim, Germany, 219–280.

Bailey JE & Ollis DF (1986) Biochemical Engineering Fundamentals. McGraw-Hill, Singapore, 460.

Kamen AA, Tom RL, Caron AW, Chavarie C, Massie B & Archambault J. (1991) Culture of insect cells in a helical ribbon impeller bioreactor. Biotechnol. Bioeng. 38: 619–628.

Martens DE, Nollen EAA, Hardeveld M, Van der Velden-de Groot CAM, De Gooijer CD, Beuvery EC & Tramper J (1995) Death rate in a small air-lift loop reactor of Vero cells grown on solid microcarriers and in macroporous microcarriers. Submitted.

Papoutsakis ET (1991) Media additives for protecting animal cells against agitation and aeration damage in bioreactors. Trends in Biotechnol. 12: 45–62.

Sänger P & Deckwer WD (1981) Liquid-solid mass transfer in aerated suspensions. Chem. Eng. J. 22: 179–186.

Sano Y, Yamaguchi N & Adachi T (1974) Mass transfer coefficients for suspended particles in agitated vessels and bubble columns. J. Chem. Eng. Japan 7: 225.

Spier RE & Griffiths B (1984) An examination of the data and concepts germane to the oxygenation of cultured animal cells. Dev. Biol. Stand. 55: 81–92.

Tramper J, De Gooijer CD & Vlak JM (1993) Scale-up considerations and bioreactor development for animal cell cultivation. In: Insect cell culture engineering (Goosen MFA, Daugulis AJ and Faulkner P, eds) Marcel Dekker, New York, USA, 139–177.

van 't Riet K & Tramper J (1991) Basic Bioreactor Design, Marcel Dekker, New York.

Verlaan P (1987) Modelling and characterization of an air-lift loop bioreactor. PhD thesis, Wageningen Agricultural University, The Netherlands.

Wijffels RH, De Gooijer CD, Schepers AW, Beuling EE, Mallée LR & Tramper J (1995) Dynamic modelling of immobilized *Nitrosimonas europaea*: implementation of diffusion limitation over expanding microcolonies. Enzyme Microb. Technol. 17: 462–471.

Wijffels RH, de Gooijer CD, Kortekaas S & Tramper J (1991) growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan: Part 2. Model evaluation. Biotechnol. Bioeng. 38: 232–240.

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Downstream processing of insect cell cultures

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Introduction

The downstream processing of insect cell cultures is highly variable, reflecting the variety of proteins which have been produced with this technology. A large number of proteins of human origin have been produced using the baculovirus/insect cell system (Luckow, 1993; O'Reilly *et al.*, 1994). They represent almost all possible localisations: nuclear, cytoplasmic, membrane spanning or secreted to the extracellular medium. The choice of a particular downstream strategy is primarily dictated by scale of operation, localisation of the target protein and recovery yield.

However, other practical criteria such as processing time, containment, manpower requirements, compatibility with sterility or sanitization and overall cost should also be considered at the design stage. In most cases, a translation of a process for the purification of proteins from recombinant or natural mammalian cell sources is quite suitable. Some issues are more specific to insect cell cultures. The culture broth usually contains a high level of baculoviruses (10^6–10^9 ml^{-1}). Thus, the background from which the protein needs to be purified contains additional viral proteins and DNA. Insect cell culture media (see paper by Ernst-Jürgen Schlaeger, pp. 57–70, this volume) also contain lipids (Cholesterol, tocopherol, fatty acids), surface active agents such as Triton X–100, Pluronic F68 (Murhammer & Goochee, 1988) and antifoams which can interfere with some downstream operations. Most large scale processes are now run in serum free, low protein media. At small scale, even if cell growth is carried out in serum containing medium, it can be replaced by a serum free medium at the start of infection (Summers & Smith, 1987; York *et al.*, 1994).

The time of harvest strongly influences product quantity and quality. The viability of the cells at the time of harvest can be as low as 30–40% (Reuveny *et al.*, 1993a,b) and therefore, half of the cell population may have lysed and released its intracellular contents into the medium. This reduces the segregation of the product into well defined compartments (either cell associated or secreted). It also introduces significant amounts of intracellular proteases (Jäger *et al.*, 1992, Grabenhorst *et al.*, 1992) and glycosidases (Bailey, 1995) into the medium. As a consequence, a cell associated protein may be found both in the cell pellet and in the conditioned medium at the time of harvest. A secreted protein may be altered by the proteolytic or glycolytic activities released from lysed cells. Obviously, an optimised production process and a careful choice of harvest time should minimise this phenomenon and keep the two compartments well separated.

On a large scale, the initial steps of downstream processing are common to all processes as illustrated in Figure 1 and are described in part 1. The first step consists of a solid-liquid separation where cells are isolated from the conditioned medium. This is required whether the protein is cell associated or secreted. Cell harvesting should be done carefully to avoid partial cell disruption by mechanical shear stress. This can lead to cell protease release which may affect a secreted protein. or may result in product loss if cell associated. Several technologies of cell harvesting are available based on centrifugation or filtration. Presently, the bioreactor configuration used for culturing has

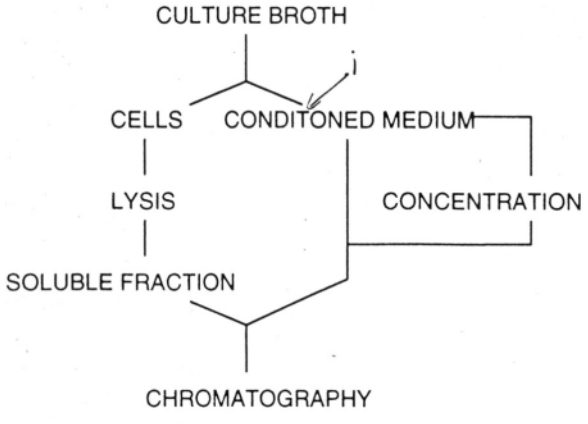

Figure 1. Downstream processing initial steps. The whole culture broth is first separated into cells and conditioned medium. For intracellular proteins (but not membrane spanning), cells are lysed, and the soluble fraction subjected to chromatography. For secreted proteins, the conditioned medium can be concentrated or directly subjected to chromatography.

little influence on the downstream processing strategy, except for special designs where the insect cells are physically retained. Examples are stationary beds (Kompier *et al*., 1991), microcapsules (King *et al*., 1989) or production of secreted proteins in perfusion reactors. In these cases, obviously the cell separation is built into the culture system.

The choice of the subsequent steps depends on the localisation of the protein (cell associated or secreted). For cell associated products, the cell paste usually goes through one or several freeze/thaw cycles to facilitate lysis. Cells are then mechanically broken or lysed in detergent, and/or hypotonic buffers. Cell debris is eliminated by another solid-liquid separation and chromatography follows. Membrane proteins represent a special class of proteins which require specific attention. Expression levels are usually much lower than soluble proteins and purification from the membrane may irreversibly damage the native structure. This may result in a pure but modified protein. For proteins secreted on large scale, the conditioned medium is concentrated 10–50 fold by ultrafiltration or precipitation induced by salts or pH. The concentrate is then processed by chromatographic techniques. In some cases, the concentration step is omitted and non-concentrated supernatant is directly processed by immuno-affinity binding (Kikuchi *et al*., 1994; Hurwitz *et al*., 1991).

Chromatography is based on separation by charge, hydrophobicity, size or affinity for a ligand (molecular recognition). Since the protein is normally properly folded and immunoreactive, many purification strategies include an immuno-affinity step. It is quite often used very early in the purification process. Epitopes used for the affinity capture may be from the protein itself (Greenfield *et al*., 1988; Pochon *et al*., 1992; Singer *et al*., 1994), from a small tag (Furlong *et al*., 1988; Clark *et al*., 1992; Herren & Pech, 1992; Haubruck *et al*., 1993; Wang *et al*., 1994) or a fusion partner (Cooke *et al*., 1994; Peng *et al*., 1993). This allows the use of the same generic method for the recovery of many different proteins. Depending on the final target purity, immuno-affinity purification may be the only necessary step (Clark *et al*., 1992).

Some enzymes are expressed at such high levels in the baculovirus/insect cell system, that they were purified to homogeneity and crystallised: bovine inositol polyphosphate 1-phosphatase (York *et al*., 1994), rat acid phosphatase (Vihko *et al*., 1993), human purple acid phosphatase (Hayman & Cox, 1994).

Expression of cytoplasmic or trans-membrane proteins can lead to aggregation, insolubilisation and consequent inactivation. Some proteins are made with only a small proportion soluble: human 5 lipoxygenase (Denis *et al*., 1991), phosphatase I subunit (Berndt & Cohen., 1990), glucocorticoid receptor (Alnemri *et al*., 1991), protein kinase C (Rankl *et al*., 1994), Na^+/H^+ antiporter (Fafournoux *et al*., 1991). Thirty percent of the transferrin receptor had non-native disulfide bridges (Domingo & Trowbridge, 1988). These non-active or insoluble species can only be restored active after solubilisation into denaturants and refolding, which renders the whole process very inefficient. Both cytoplasmic and secreted proteins are subject to proteolytic degradation(McGlynn *et al*., 1992; Jäger *et al*., 1992; Grabenhorst *et al*., 1992) which complicates the purification.

Some recombinant proteins can also be produced in larvae instead of cells in culture. Purification from larvae is not specifically addressed here. It has been published for cytoplasmic proteins such as v-sis (Morishita & Maeda, 1991), adenosine deaminase (Medin *et al*., 1990) and secreted proteins such as human α-interferon (Maeda & Kawai, 1985), mouse interleukin–3 (Miyajima *et al*., 1987), feline interferon (Nakamura *et al*., 1992) and apolipoprotein E (Gretch *et al*., 1991). For secreted proteins, downstream processing of the larvae hemolymph is identical to that of the cell culture supernatant.

Wild type (or recombined with a toxic gene) baculovirus is also produced at large scale for use as

biopesticide (Belisle *et al.*, 1991), but the downstream treatment applied to these productions is outside the scope of this chapter.

In this chapter, we will first review the initial steps which are common to most purification processes. In a second part, we will describe specific case studies which illustrate purification of three different types of proteins: cytoplasmic, secreted, and transmembrane.

Part 1: Initial steps common to all processes

Cell separation

Several technologies are available to separate cells from the conditioned medium. The same technologies are also used for recycling the cells into the reactor and operate it in perfusion (Tokashiki & Takamatsu, 1993). The mechanical fragility of the infected cells is one of the critical issues of cell separation.

Batch centrifugation

The simplest technique, batch centrifugation is quite suitable for small scale volume harvesting (Lazarte *et al.*, 1992). A typical 300xg force (15–30 min) should be used to prevent cell breakage, particularly if the target protein is cell associated (Van Reis *et al.*, 1991). Because of the relatively low g force, the resulting supernatant still contains a lot of particles and cell debris. Therefore, if the supernatant needs to be further processed, it should be filtered through a tangential flow microfiltration unit, pore size 0.22 μm.

Continuous centrifugation

Centrifugation can be achieved on a larger scale by continuous feed to the rotor. Gronvick *et al.* (1989) designed and used a system (Alfa-Laval Centritech) with a rotating plastic bladder. The culture broth is fed at a rate of 40 l/h to a plastic bladder (capacity 490 ml) which rotates at approximately 1200 rpm (300xg) (Hodgson, 1991). Cells migrate quickly to the bladder wall by centrifugal force, and the cell free supernatant flows out of the device into a harvest tank. The system has been used suscessfully for harvesting mammalian cells (Apelman, 1992) and is applicable to insect cell separation (Overton & Kost, 1995). The principle of continuous feed centrifugation can be applied to a titane rotor (Heraeus Contifuge, 300 ml capacity) replacing the need for a bladder. Here rotation speeds can be as high as 15000 rpm. Riese *et al.* (1994) used this sytem for the separation of hybridomas with a flow rate of 300 ml min^{-1}. Schlaeger *et al.* (1992) separated infected *Sf9* cells and we have repeatedly achieved very good separation of infected insect cell cultures (*Sf9* and *Tn5* lines) at flow rates up to 18 l/h and with limited loss of cell viability despite a high g force (1000–1500xg).

Microfiltration

Tangential flow or crossflow filtration (flat, spiral membrane or hollow fibre) offers the largest flexibility and is amenable to scale-up. Mawson (1993) recently reviewed its application to the separation of microbial cells. A large variety of membranes and equipment is available. Cellulose esters and polysulphone are the most commonly used materials. We have used porosities between 0.1 and 0.6 μm with good separation efficiency. In case of fouling, the filter can usually be quickly cleaned or back-flushed, and put back into operation. At the end of the filtration, the membranes should be cleaned by chemicals such as NaOH (0.1 N); NaCl (1M), NaOCl (100 ppm) or urea (5 M), but compatibility with the membrane material and equipment should be checked with the manufacturer. Fouling should be avoided also because it usually leads to retention of protein species by molecular sieving. Shiloach *et al.* (1986) reported a comparative study of three different filters for harvesting mammalian cells. Van Reis *et al.* (1991) designed a large capacity system (5000 l/h) using 0.2 μm hollow fibers for separating recombinant CHO cells. Barkhem *et al.* (1992) described the use of a flat membrane system with 0.45 μm porosity and 2 m^2 filtration area (Millipore Prostak) for harvesting cells from 100 l bioreactors at a rate of 60 l/h.

Maiorella *et al.* (1991) used mammalian and *Sf9* cells as models for establishing optimal conditions of circulation flow rate, transmembrane pressure and wall shear rate for the operation of tangential flow filters. They recommended to operate below a critical average wall shear rate of 3000 s^{-1} at which cell damage starts.

Expanded beds

A novel technique of direct capture of the product by expanded beds was recently developed (Streamline from Pharmacia). It has been evaluated with yeast, bacteria, and hybridomas (Schmidt *et al.*, 1994) or CHO cells (Erickson *et al.*, 1994) but no data have been published on its applicability to insect cell culture processing. These techniques are quite attractive since they

allow direct processing of the whole broth. This eliminates the initial steps associated with cell separation and speeds up the entire downstream processing.

Crude supernatant concentration

This step can be handled in very similar conditions to the concentration of conditioned medium of mammalian cell origin (Menge *et al*., 1987; Tolbert & Prior, 1988; Goodall *et al*., 1992). For small scale operations, concentration can be carried out by dead-end ultrafiltration in stirred cells with low protein capacity binding membranes (Goodall *et al.*, 1992). Concentration can also be achieved by ammonium sulfate precipitation (Vissavajjhala & Ross, 1990). This technique combines concentration and partial purification. An alkaline pH shift to 8.0 also allows partial purification by precipitating contaminating proteins and viruses (Hayman & Cox, 1994; Nutt *et al.*, 1991). For large scale, tangential flow ultrafiltration systems provide a large surface area of membrane. This technique has been applied successfully to the concentration of monoclonal antibody from hybridoma conditioned medium (Prior *et al.*, 1989). The membranes are built in flat plate, spirals, hollow fibre or tubular configurations. Some purification is also achieved by size exclusion but in practice the resolution is poor. Schlaeger *et al.* (1992) separated the cells by a continuous centrifuge and concentrated the 60 l culture supernatant 10–20 fold by a tangential flow ultrafiltration system (Amicon SP20) with a filtration flow rate around 100 l/hr. The molecular weight cut-off of the hollow fiber membrane (2 m^2, 10 kDa), allowed complete retention of the secreted ectodomain of TNF receptors (55 and 75 kDa). Concentration by ultrafiltration can be combined with buffer exchange by diafiltration which allows to reach the desired type of buffer, ionic strength, and pH for the next step (Quelle *et al.*, 1989). It can be applied to affinity purification in solution by using a high molecular weight affinity ligand. The protein-ligand complex is thus retained by an ultrafiltration membrane of high molecular weight cut-off. Luong *et al. (*1987) described such a system to purify trypsin from porcine pancreatic extracts. Affinity purification can also be combined with ultrafiltration with membranes where the ligand is covalently bound. Brandt *et al.* (1988) reported the purification of Fibronectin from human plasma by gelatin covalently attached to cross-linked agarose and Nachman *et al.* (1992) described membranes grafted with the soluble domain of the IL–2 receptor for the purification of recombinant IL–2 (mammalian cell origin). Kroner *et al.* (1992) reported the purification of malate dehydrogenase from *Escherichia coli* and *Saccharomyces cerevisiae* as a model system and Reif *et al.* (1994) evaluated metal affinity membranes which should be applicable to poly-His tagged proteins.

Cell breakage

Typically cell breakage techniques commonly used for disruption of microorganisms have been applied to the disruption of insect cells with little modification (Table 1).

Homogenisation

One of the most common and simplest methods is mechanical homogenisation. Due to the low shear imparted, Dounce homogenisers have been the most popular choice to homogenise insect cells in an initial step to recover recombinant protein (Lowery *et al*., 1991; Chang *et al.*, 1992; McGlynn *et al*., 1992; Chen *et al.,* 1993) Typically the cells are first subjected to hypotonic shock (5–50 mM Tris-HCl, pH 7.6). The hypotonic solution causes swelling of the cells which renders them more susceptible to mechanical cell lysis. The extent of cell breakage can be monitored by phase contrast microscopy. Lysates obtained from homogenisation protocols are usually clarified by centrifugation at 10 000xg for 5 min.

Freeze-thaw

Freeze-thawing is a simple yet very efficient means of cell lysis, however, the successful application of this technique depends on the stability of the recombinant protein after one or more consecutive freeze-thaws. Wickham *et al.*, (1991) successfully employed freeze thawing with three different cell lines including *Tn368, Sf21* and *Sf*9 as a one step protocol for extraction of β-gal. Hink *et al.*, (1991) applied the method for the release of the same enzyme in 23 different cell lines. Repetitive freeze-thawing has also been reported. Prehaud *et al.* (1990) disrupted *Sf*9 cells for purifying a rabies virus nucleoprotein (N) by three cycles of freezing in solid dry ice followed by immediate thawing at 37 °C. The cell debris was used as an immunogen for the production of antibodies against the recombinant protein in mice. Additionally freeze thawing has also been used as a pre-treatment in combination with other cell disruption steps (Chen *et al.*, 1992; McGlynn *et al.*, 1992; Berndt & Cohen, 1990).

Table 1. Cell disruption methods

Method	Cell line	Recombinant protein	References
Homogenisation	*Sf9*	Alzheimer's amyloid precursor	Lowery *et al.*, 1991
	Sf9	Human epidermal growth factor receptor	McGlynn *et al.*, 1992
	Sf9	Human androgen receptor	Chang *et al.*, 1992
	Sf21	Two subunit of farnesyltransferase	Chen *et al.*, 1993
	Sf9	Protein tyrosine kinase pp60c-src	Budde *et al.*, 1993
Freeze-thaw	*Tn368, Sf21, Sf9*	β-galactosidase	Wickham *et al.*, 1991
	23 cell lines	β-galactosidase	Hink *et al.*, 1991
Sonication	*Sf9*	EBV alkaline DNase	Baylis *et al.*, 1991
	Tn5	G protein	Graber *et al.*, 1992
	Sf9	VP2H	Wang *et al.*, 1993
	Sf9	CFTR	Peng *et al.*, 1993
	Sf9	Haemagglutinin	Possee, 1986
	Sf9	Protein kinase C	Rankl *et al.*, 1994
Hypotonic shock	*Sf9*	rabies nucleoprotein (N)	Prehaud *et al.*, 1990
	Sf9	human poly (ADP-ribose) polymerase	Giner *et al.*, 1992
Detergent lysis	*Sf9*	HIV gp160	Wells & Compans, 1990
	Sf9	v-src, c-src	Park *et al.*, 1992
	Sf9	HSV type 2 glycoprotein D	Landolfi *et al.*, 1993
N_2 cavitation	*Sf9*	G protein subunits	Graber *et al.*, 1992
	Sf9	Adenylylcyclase	Tang *et al.*, 1991

Osmotic shock

Hypotonic shock aids disruption of cells and can cause osmotic damage to the nucleus and organelles and is therefore very useful if the protein of interest is located in these areas. Prehaud *el al.* (1990) released intranuclear rabies nucleoprotein by resuspension of infected insect cell pellets in 0.5 mM Tris-HCl (pH 7.5) on ice for 30 minutes. Similarly Giner *et al.* (1992) achieved one step lysis using a hypotonic buffer for the release of full-length human poly(ADP-ribose)polymerase (PARP) in baculovirus infected *Sf9* cells.

Sonication

Sonication (ultrasonic disintegration) has been used mainly as one step in a cascade of other insect cell disruption steps to ensure cell lysis (Graber *et al.*, 1992; Wang *et al.*, 1994; Denis *et al.*, 1991). The cells are sonicated continuously or with a number of pulses of sonic energy of a few Watts (Stauffer *et al.*, 1991; Peng *et al.*, 1993) after other homogenisation or freeze thaw procedures. Baylis *et al.* (1991) used hypotonic buffer followed by gentle sonication at 0 °C using a water bath sonicator to release EBV alkaline Dnase from *Sf9* cells. A combination of flash freezing at –80 °C, probe sonication in hypotonic buffer and centrifugation followed by a second round of sonication was required to release protein kinase-C from insect cells (Rankl *et al.*, 1994). Using sonication as a one step process would require careful consideration of parameters including the duration and number of consecutive pulses as well as the distribution of the integrity of membrane states found at various times post-infection within the cell population.

Gas cavitation

Gas cavitation has also been reported for disruption of baculovirus infected insect cells. Typically this procedure involves the suspension of cells in a homogenisation buffer in a nitrogen containing metal cylinder for 5–30 min at high pressures (600 psi and above). On return to atmospheric pressure, the nitrogen gas dissolved in the cytoplasm is released aiding cell disruption by rapid expulsion of the cells from the cylinder via a narrow orifice. Typically organelles, including the nucleus, are released intact using this method. Graber *et al.* (1992) thawed frozen harvested cells into homogenisation buffer and burst them by N_2 cavitation (300–600 psi for 20 min). The intact nuclei and cell debris were removed by low speed centrifugation leaving the soluble cell extract that contained two subunits of recombinant G proteins. Tang *et al.* (1991) also

employed the procedure of N_2 cavitation but retained the cell membrane pellet for mechanical homogenisation, followed by detergent extraction, to release a recombinant calmodulin-activated (Type 1) adenylcyclase.

Cell breakage for membrane proteins

Disruption of insect cells for the purification of membrane proteins involves special techniques. There are basically two ways to purify transmembrane receptors from recombinant insect cells (Figure 2).

1) Preparation of membranes from cell lysates followed by detergent extraction.

   Separation of the membrane fraction is achieved by continuous or discontinuous sucrose density gradient (Stauffer *et al.*, 1991) or by ethanol precipitation (Pochon *et al.*, 1992). Alternatively, cell lysis is followed by differential centrifugation of the membrane components (Evans, 1978; Sheeler, 1981; Parker *et al.*, 1991; Reilander *et al.*, 1991).

   For these methods, based on the enrichment of the preparation with membranes, the aim is to conserve the membranes integrity as much as possible in a homogeneous preparation. In all cases the preparation starts by cell breakage followed by a centrifugation step which isolates the insoluble fraction containing the membranes.

2) Whole cell detergent extraction.

   This method combines breakage and membrane protein extraction in a single step. Cationic, anionic or zwitterionic surfactants or detergents have been used for recovery of intracellular proteins of the refractile bodies and cytoplasmic aggregates found in recombinant bacterial cultures. The same has been applied to insect cell cultures. Researchers have employed the process of surfactant solubilisation as the primary step in purification and isolation of some membrane associated proteins (Domingo & Trowbridge, 1988; Peng *et al.*, 1993). Proteins have been extracted from the membrane lipid bilayer of baculovirus infected insect cells by insertion of their hydrophobic regions into detergent micelles. Wells & Compans (1990) purified the envelope glycoprotein of HIV (gp160) and used a lysis buffer including 1% triton X–100, 1% Deoxycholate and 0.1% SDS as detergents. Pelleted cells were resuspended in this buffer, incubated on ice for 15 min and microfuged before being used for immune precipitation. Similarly, Landolfi *et al.* (1993) used surfactant solubilisation for the initial purification of herpes simplex virus type 2 glycoprotein D.

   Some published examples describe cell disruption by detergents only (Narum *et al.*, 1993; Webb *et al.*, 1989; Paul *et al.*, 1990). The detergent cell extract contains both the insoluble and the soluble proteins and subsequently requires additional purification steps to achieve good purity of the recombinant membrane protein. A disadvantage associated with detergents is that membrane-bound proteases may be activated during prolonged procedures, although the effect has not yet been reported in insect cells.

Part 2: Protein specific processes: case studies

This part illustrates purifications of proteins routinely carried out at large scale in our laboratories. They were chosen to represent three different types of proteins namely cytoplasmic, secreted and embedded in the cytoplasmic membrane. The aim is to detail the experimental procedures used and the results obtained in each case. These methods should be equally applicable to other recombinant proteins.

*Case study 1: **Gαq**, model of cytoplasmic protein purification*

Heterotrimeric guanine nucleotide-dependent regulatory proteins (G proteins) are an essential part of the signal transduction pathways which mediate the cell's response to hormones and neuromodulators. The Gq class of G proteins includes **Gαq** from mouse, drosophila and squid photoreceptor membrane. Members of this class mediate the hormonal stimulation of membrane phosphoinositides by **PLC-β1**, **PLC-β2** and **PLC-β3** (Smrcka *et al.*, 1991; Gutowski *et al.*, 1991; Taylor *et al.*, 1991). A variety of G protein subunits have been purified from baculovirus infected insect cells by several groups (Graber *et al.*, 1992; Robishaw *et al.*, 1992; Labrecque *et al.*, 1992; Hepler et al., 1993; Jones *et al.*, 1993; Ueda *et al.*, 1994; Singer *et al.*, 1994). We had previously purified **Gαq** from mouse brain and shown it to stimulate the activity of polyphosphoinositide-specific Phospholipase C **(PI-PLC)-β1**. The purpose of this work was to obtain large amounts of protein from recombinant sources for futher studies and characterization. **Gαq** cDNA from mouse brain (Strathmann & Simon, 1990) was cloned into a baculovirus expression vector downstream of the polyhedrin promotor using standard methods (Chapter

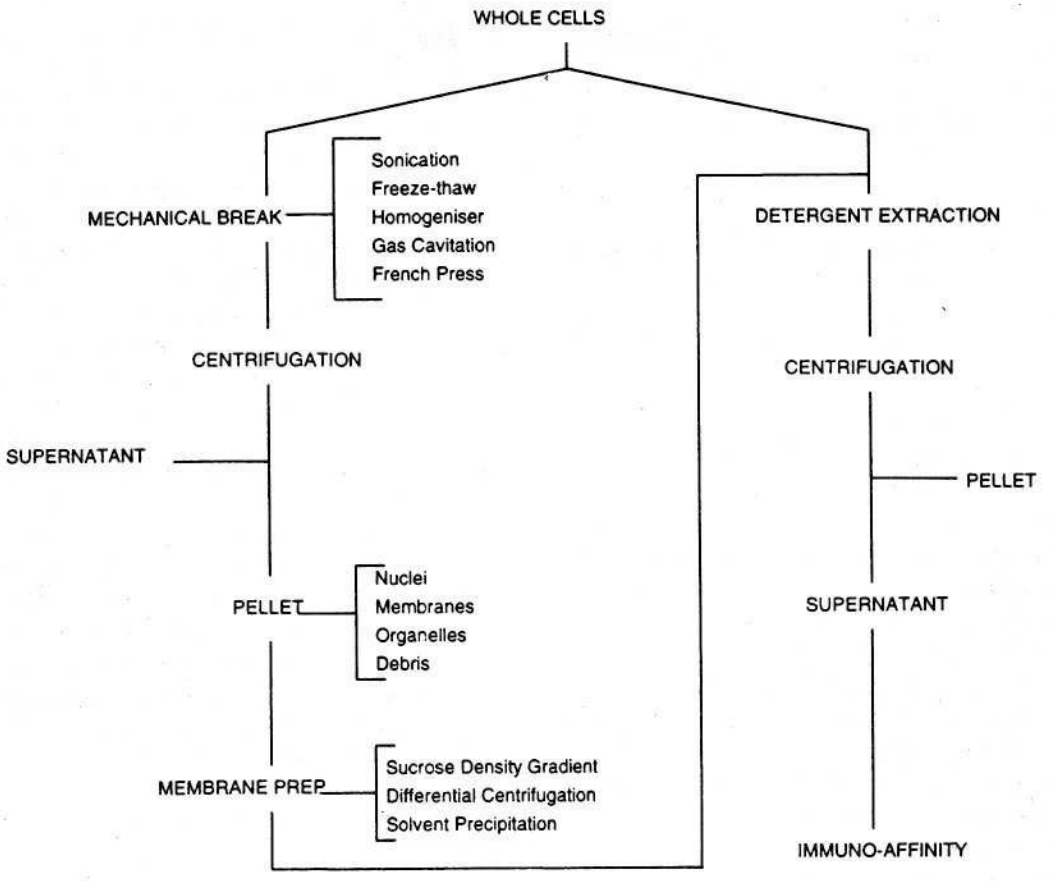

Figure 2. Purification of transmembrane proteins. Purification of transmembrane proteins can be achieved by two main routes: whole cell detergent extraction, or preparation of a membrane fraction after mechanical cell break.

Table 2. Purification of recombinant Gαq. 6.5 g wet weight of *Sf9* cells were used for the purification described

Fraction	Volume (ml)	Protein (mg)	[^{3}H]GTP (nmol)	Binding (nmol mg^{-1})	Yield (%)
Sonicated cells	100	285	144	0.05	100
100 000xg supernatant	100	114	124	0.11	86
DEAE	9.5	30	15.1	0.34	10
Hydroxyapatite	10	4	3.7	0.93	3
Mono Q	1.5	1.7	2	1.58	1.5

9, this issue). Upon infection of *Sf9* cells, a 42 kDa band showed up on a SDS-PAGE gel stained with Coomassie blue. Western blot analysis of uninfected and infected cells showed the presence of endogenous Gαq in *Sf9* cells which has made the purification and characterisation of the recombinant protein more difficult.

The cell extraction protocol is modified from that of Graber *et al.* (992). All the extraction steps were done at 4 °C. Cells in the culture broth (2×10^6 cells ml^{-1}, 4 or 15 litre reactors) were harvested by low speed centrifugation (200xg, 15 min). The resulting pellet was immediately frozen at –80 °C and stored for a few days. The cells were thawed in 15 × their wet weight of homogenisation buffer (10 mM Tris-HCl, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 mM NaF, 10 μM $AlCl_3$,

10 μM GDP, pH 8.0) and broken by 2× 30 s sonication. GDP was included in the homogenisation buffer to keep the protein correctly folded and stable. Sonicated cells were centrifuged for 90 min at 100 000xg to remove unbroken nuclei and cell debris. The soluble cell protein fraction was loaded onto a 2.5 × 5 cm DEAE column equilibrated in TED buffer (50 mM Tris-HCl, 0.02 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, pH 8.0) and eluted with a gradient from 0–0.7 M NaCl in TED using a flow rate of 1.2 ml min^{-1}. Column fractions were assayed for GTP binding activity using [^{3}H] GTP. Each assay tube contained 10 μl sample in a total volume of 150 μl incubation buffer (75 mM Na-HEPES, pH 8.0, 50 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 0.5 mM ATP and 1 μM GTP (4 μCi of [^{3}H] GTP ml^{-1}). Tubes were incubated for 20 min at 30 °C and binding terminated by addition of 1 ml ice-cold wash buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 25 mM $MgCl_2$, pH 8.0) followed by rapid filtration through nitrocellulose filters. Those fractions with binding activity were pooled, diluted with 4 volumes of buffer containing 10 mM K_2HPO_4, 10 mM Tris-HCl, pH 8.0 and loaded onto a 1.5×4 cm hydroxyapatite column equilibrated in the same buffer. The column was eluted from 0–300 mM K_2HPO_4 with a 80 min linear gradient and a flow rate of 0.8 ml min^{-1}. The fractions containing GTP binding activity were loaded onto a HR 5/5 MonoQ column equilibrated with 25 mM Tris-HCl, 0.02 mM EDTA, 1 mM DTT and 1 mM $MgCl_2$ and eluted from 0–1 M NaCl for 100 min with a flow rate of 0.8 ml min^{-1}. The proteins were well separated and the GTP activity could be traced to one fraction. The protein was purified to homogeneity as shown on a SDS-PAGE gel stained with Coomassie Blue (Figure 3). However, because of the number of steps involved in our purification scheme, the final yield was low and on average 10 fold less than published data (Graber *et al.*, 1992). The activity of the pure Gaq protein was confirmed by a PLC activation assay using the method described by Gutowski *et al.* (1991).

Case study 2: E-selectin, model of a secreted protein

E-selectin is a cell adhesion molecule which mediates the initial 'rolling' of neutrophils on endothelium at a site of inflammation by interaction with the tetrasaccharide ligand sialyl Lewis X presented on endothelial cells (Springer & Laski, 1991). We previously reported (Cavegn *et al.*, 1992) the production of a recombinant form of the protein in which the C-terminal membrane-spanning region was replaced with two consensus domains from *Staphylococcus aureus* protein A termed 'zz' (Löwenadler *et al.*, 1987).

The modified E-selectin (E-selectin-zz,) was cloned into baculovirus to give a secreted protein, and after culture was purified using immobilised IgG to capture the protein by interaction betweeen the Fc domain of IgG and the zz domains of E-selectin-zz.

Trichoplusia ni cells (which were adapted to grow in suspension culture) were cultured in a 36 litre air-lift fermenter using Excell 401 medium supplemented with 5% foetal calf serum. The cells were infected at a density of 2.8×10^6 cells ml^{-1} with baculovirus (Multiplicity of infection = 1), and harvested 3 days after infection (3.1×10^6 cells ml^{-1}, 93% viable). The cells and the medium (36 litres) were separated using a tangential flow membrane apparatus (Millipore Prostak Dual-pump mammalian harvest system) fitted with three 10-stack modules (PSGVAG101, 0.22 μm) of 0.84 m^2 each. The recirculation rate was 6.7 l min^{-1}, the permeate flow was 0.63 l min^{-1} (flux 15 l hr^{-1} m^{-2}) and the transmembrane pressure was 0.2 Bar. After washing the retentate with phosphate buffered saline, the total volume of clarified supernatant was 40 litres. A cocktail of protease inhibitors (benzamidine 1 mM, PMSF 1 mM, leupeptin 5 mg l^{-1}, SBTI 5 mg l^{-1} and pepstatin A 5 mg l^{-1}) and sodium azide (5 mg l^{-1}) were then added to prevent proteolysis and bacterial growth, and the supernatant was stored at 4 °C prior to processing.

The E-selectin-zz was purified (room temperature throughout) in two 20 litre batches using a Quantasep instrument (Sepragen). The supernatant was loaded onto human IgG-agarose (from ACL, packed in a 100 ml radial flow column) at a flow rate of 100 ml min^{-1}. After the column had been loaded and washed with buffer (20 mM sodium phosphate pH 7.2, 0.5 M NaCl) the E-selectin-zz was eluted with 3 M ammonium thiocyanate. The pooled material was then desalted on a 3 1 Sephadex G25 column using sodium phosphate buffer (without NaCl) at a flow rate of 300 ml min^{-1}. The entire purification was run unattended by using the chromatography control software. The purity of the protein was determined by gel electrophoresis. Adhesion of HL60 cells to E-selectin-zz bound to IgG coated wells was used to monitor biological activity.

The elution profile from the IgG column is shown in Figure 4. The pooled material was loaded directly onto the G25 column to remove the ammonium thiocyanate. This compound absorbs strongly at 280 nm and is responsible for the absorbance after the pro-

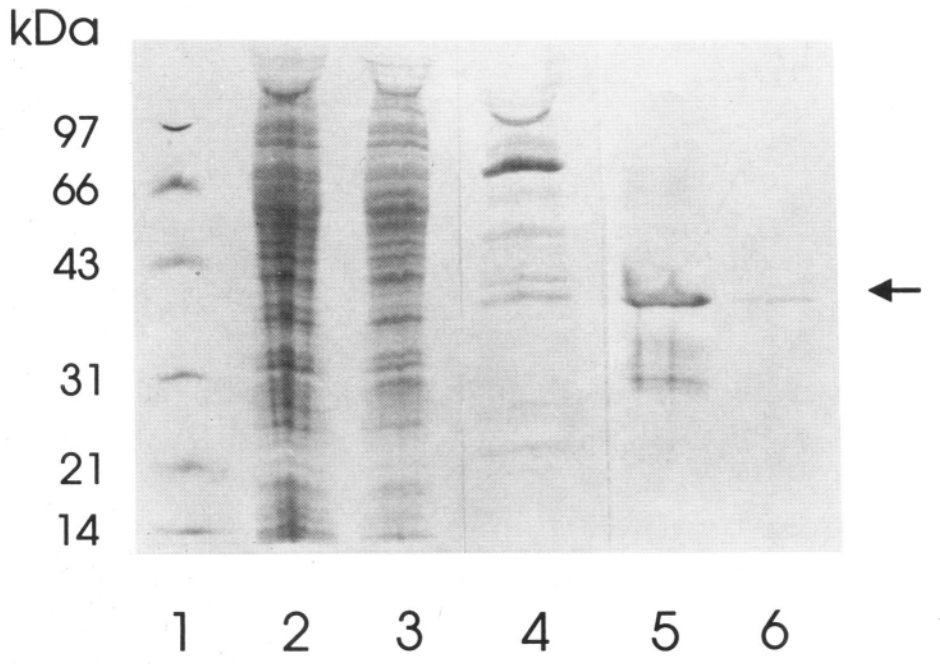

Figure 3. Purification of recombinant GαQ. The protein was purified to homogeneity. A SDS-PAGE gel under reducing conditions was run and stained with Coomassie Brilliant Blue R250 after electrophoresis. Samples are homogenised cells (*lane 1*), 100 000xg supernatant (*lane 2*), DEAE pool (*lane 3*), hydroxyapatite pool (*lane 4*), Mono Q pool (*lane 5*), Molecular weight markers (*lane 6*). The arrow points to the purified GαQ.

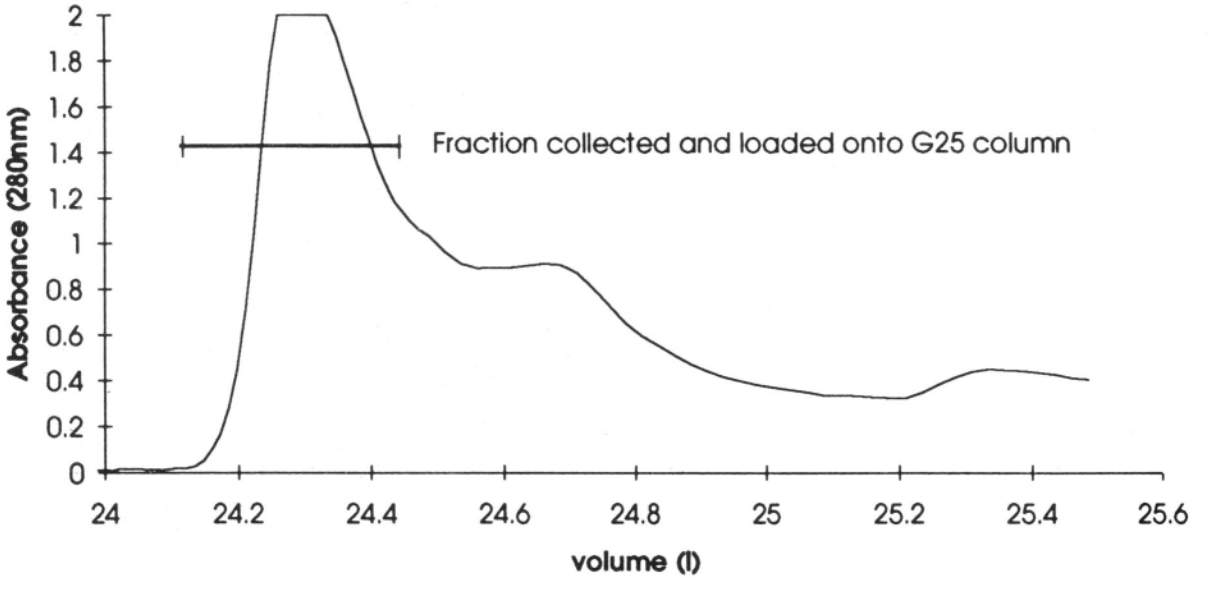

Figure 4. Elution profile of the IgG agarose column. After cell separation by tangential flow microfiltration, 20 l of the cell free medium were loaded onto an IgG agarose 100 ml column. The column was washed with 20 mM sodium phosphate pH 7.2, 0.5 M NaCl and eluted with 3 M ammonium thiocyanate. The automatic chromatography control system monitored purification by absorbance at OD280nm.

Table 3. Summary of purification runs for E-selectin-zz

Sample	Protein conc. (mg ml^{-1})	Vol. (l)	Protein (g)
Run 1			
Crude supernatant	1.1	20	22
IgG column void	1.05	20	21
G25 column pool	0.24	0.275	0.066
Run 2			
Crude supernatant	1.08	20	21.6
IgG column void	0.81	20	16.2
G25 column pool	0.11	0.42	0.046

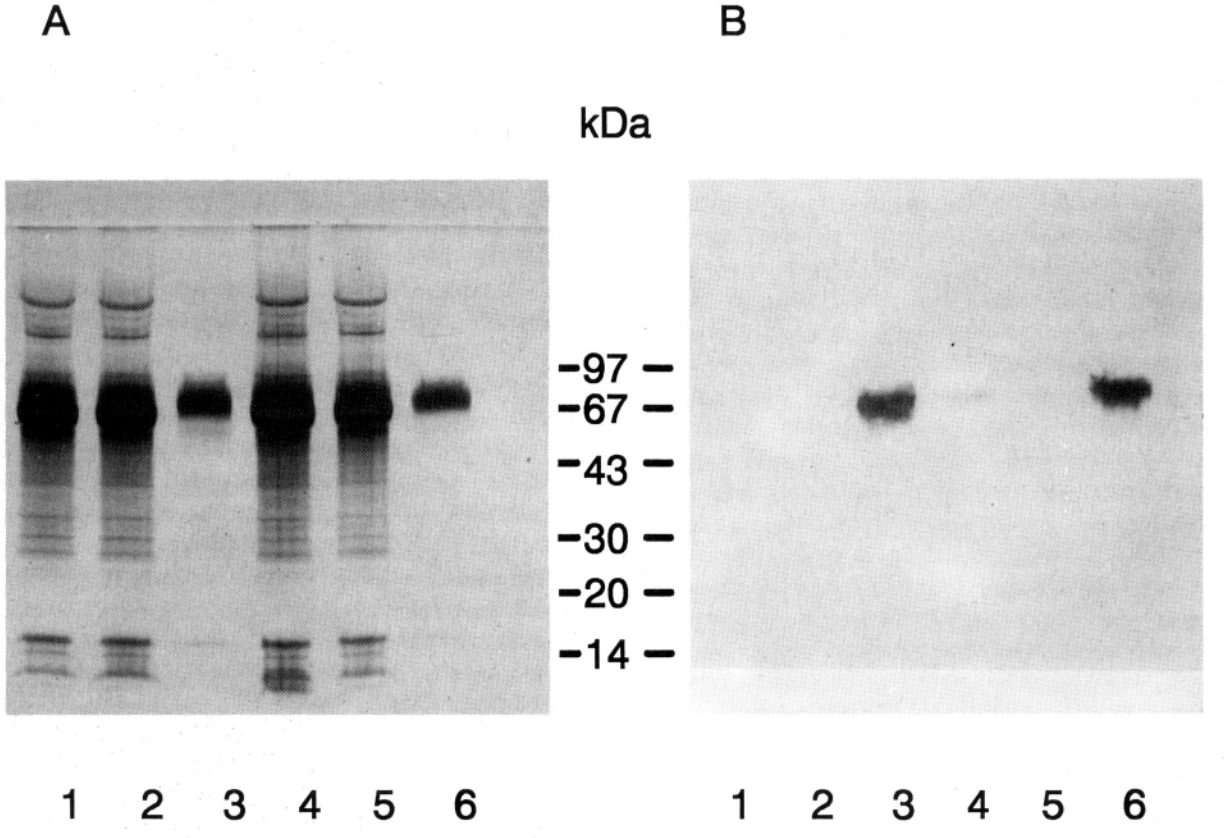

Figure 5. Gel analysis of the purification of E selectin-zz. The protein was purified to homogeneity. Two different runs are shown on a 8–25% SDS-PAGE gel (reducing conditions), silver stained (Panel A). Samples are Run 1 Load (*lane 1*), Run 1 Void fraction (*lane 2*), Run 1 Pool of pure fractions (*lane 3*), Run 2 Load (*lane 4*), Run 2 Void fraction (*lane 5*), Run 2 Pool of pure fractions (*lane 6*). An identical gel was used for a Western Blot probed with an alkaline phosphatase-IgG conjugate. Samples are the same as in Panel A.

tein peak has eluted. The purification is summarised in Table 3 and the analysis by gel electrophoresis and silver staining is shown in Figure 5 (panel A). The main protein band in the load and void fractions was albumin derived from the culture medium. Analysis of the column fractions on an 8–25% reducing SDS gel (Pharmacia Phastgel) followed by silver staining indicated that the protein in the final pool was >95% pure.

On a similar gel, the proteins were transferred to PVDF membrane and probed with an alkaline phosphatase-IgG conjugate (Figure 5, panel B). E-selectin-zz could be detected as a weak band in the load sample, but not in the void fraction. As expected, a strongly staining band could be seen in the pool. Using the biological assay, the E-selectin-zz activity could not be detected in the load (or the void) fraction, but in the G25 pool activity diluted out to 1/128 without loss of signal.

The identity of the protein from a similar purification scheme was confirmed by N-terminal sequence analysis and this also showed that the signal peptide was fully removed. The mass of the protein as determined by MALDI-TOF spectroscopy was centred around 70 kDa, but the signal peak was broad, indicating sample heterogeneity which was later confirmed to be due to glycosylation variants (data not shown). The two step purification scheme, involving IgG capture then desalting was shown to give material suitable for high throughput screening, biochemical studies and structural work (Cooke *et al*., 1994).

This study shows the power of using an affinity tag for the purification of a secreted protein. Significant quantities of the E-selectin-zz were purified using an extremely straightforward purification scheme. The scheme was automated, and the scale was matched conveniently to the fermentation. The E-selectin-zz obtained was active in the biological assay, was >95% pure and usable for all the applications required. The same purification strategy was applied to the recovery of other adhesion molecules (ICAM, VCAM) fused to zz domains.

Case Study 3: Purification of CD23, model of a transmembrane protein

CD23 is a type II single transmembrane receptor found at the surface of many hemopoietic cells. It is the low affinity receptor for IgE but other biological activities related to B cell proliferation and antigen presentation have been described for this molecule (Aubry *et al*., 1992). In our search for its ligand, now identified as CD21, we had to purify CD23 from infected *Sf9* cells on a large scale for incorporation into fluorescent liposomes. In order to identify CD21 as the CD23 ligand, many cell lines were screened for their ability to bind to CD23 liposomes (Pochon *et al*., 1992).

Most of the published work on transmembrane proteins expression in insect cells focuses on the expression and the characterisation of recombinant receptors on insect cell surface. Membrane preparations or whole cell extracts have been used without any purification for functional or binding studies. This kind of approach is only suitable for high affinity receptors and high density expression at the cell surface (Jensen *et al*., 1992; Greenfield *et al.*, 1988). Characterisation of *in vitro* activity of detergent solubilized transmembrane proteins is also possible when the protein contains a domain that possess enzymatic activity (Li *et al*., 1992). Other activity characterisation studies require a highly purified receptor and reconstitution of the activity in artificial membranes (Parker *et al.*, 1991; Reiländer *et al.*, 1991). When the recombinant receptor is expressed at very low level at the cell surface, any contamination by other proteins will have a disproportional effect on the purity. To obtain the best purity, it is advisable to start from an enriched membrane fraction.

Therefore our strategy was to first disrupt the insect cells and prepare a fraction enriched in plasma membranes. To reduce the number of purification of steps we used affinity chromatography in detergent with an anti-CD23 antibody. The protein was then reconstituted in artificial membranes by incorporation into liposomes.

Cell disruption

Insect cells were found more difficult to break than mammalian cells for our purpose. Breakage by sonication or by mechanical shearing, routinely used for mammalian cells, was not sufficient to break all the cells. We obtained a very good breakage efficiency using a French pressure cell under the same conditions used to break *Escherichia coli* cells (Wingfield *et al.*, 1987). Fresh or frozen cells were suspended in absence of detergent in hypotonic buffer, 10 mM Tris/HCl pH 7.8, with protease inhibitors (1 mM TLCK, 1 mM benzamidine, 1 mM PMSF, 10 mM iodoacetamide). The cell suspension was then passed two or three times through a French pressure cell at 18 000 psi. Cell breakage was monitored by microscopic observation after addition of 0.1% Trypan blue. The cell lysate was then diluted two fold in 10 mM Tris/HCl pH 7.8 buffer containing 0.1M NaCl, protease inhibitors (same composition as above) and 0.5 M sucrose and the solution was centrifuged at 20 000xg for 60 min and supernatant wasdiscarded.

Preparation of an enriched membrane fraction

Four methods (sucrose density gradient, differential centrifugation, Triton X-114 phase partitioning and ethanol precipitation) were tested to purify the recom-

binant transmembrane CD23 receptor from *Sf9* insect cells. They all aimed at a preparation enriched in membranes, starting form the cell lysate pellet. After each preparation, the membrane fraction was purified by immune-affinity as described by Pochon *et al.* (1992). Briefly, the membranes were extracted in 1% Triton X–100 detergent and the insoluble material was separated by centrifugation at 150 000xg for 60 min. The supernatant was immunopurified on a MAb25-Affigel 10 column (MAb25, an anti-CD23 monoclonal antibody, was coupled at 3 mg per ml resin) with constant recycling overnight. The immunoaffinity column was washed with PBS containing 1% Triton X–100 and 100 mM NaCl, then with PBS buffer containing 0.1% Triton X–100. In the third wash, the Triton X–100 detergent was replaced by 50 mM Octyl-β-glucopyranoside (OGP), a dialysable detergent. CD23 was finally eluted with PBS buffer pH 6.5 containing 50 mM OGP,and 3 M NH_4SCN. The protein was desalted and further purified on a Superdex–200 gel filtration column equilibrated in PBS buffer containing 50 mM OGP.

Sucrose density gradient

Centrifugation of the cell lysate on a sucrose density gradient yielded two fractions which were enriched in CD23 membranes: phase 1 in 28% w/w sucrose and phase 2 in 42% w/w sucrose. Both phases were diluted separately, centrifuged and extracted with 1% Triton X–100, prior to affinity purification. Analysis by SDS-PAGE and western blotting using a polyclonal anti-CD23 antibody showed that the protein isolated from phase 1 had a greater degree of purity than that isolated from phase 2 (data not shown). The receptor was then incorporated into fluorescent liposomes and the activity measured by the ability of the liposomes to bind to the RPMI 8226 cell line expressing CD21 at its surface. Both preparations were active. Using this method, the purification yield was around 18 $\mu g\ l^{-1}$ cell culture (10^9 cells l^{-1}). The major problem with this method was the poly-dispersion of the membrane vesicles over the gradient. Although we obtained a slight enrichment of the phases 1 and 2 with CD23 positive membranes, the rest of the gradient also contained traces of CD23 receptor which were discarded.

Membrane preparation by differential centrifugation

This method is routinely used with mammalian cells to isolate membranes from specific cell compartments and was reviewed by Findlay & Evans (1987). Several membrane enriched fractions at various densities were obtained, and individually extracted with 1% Triton X–100. The insoluble material was removed by centrifugation and the soluble extract was affinity purified as described above. The western blotting analysis of the various fractions shows that CD23 was found distributed in all cell compartments, both in unbroken cells and in isolated nuclei, endoplasmic reticulum and plasma membranes. Using this method, the final purification yield was 7 $\mu g\ l^{-1}$ cell culture (10^9 cells l^{-1}).

Phase partitioning in Triton X–114

Triton X–114 detergent aggregates into micelles when the temperature is raised above 20°C. This property was exploited to isolate a membrane rich fraction from the cell lysate. Insect cells were lysed at 4 °C with a buffer containing 1% Triton X–114 and the insoluble material removed by centrifugation (100 000xg 30 min). Upon heating the extract at 37 °C for 10 min, two phases formed. After centrifugation, the cloudy detergent phase enriched in membrane proteins was collected (Bordier, 1981). This method did not result in any purification of CD23 as judged by SDS-PAGE and Western Blot analysis. It was thus not pursued further.

Whole membrane preparation by ethanol precipitation

The cell lysate pellet was washed by several centrifugations (20 000xg, 60 min) in 10 mM Tris/HCl pH 7.8 buffer containing 0.1 M NaCl, protease inhibitors (and 0.5 M sucrose. The pellet was resuspended in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and protease inhibitors (same composition as above). It was then precipitated with an equal volume of cold ethanol (–20 °C), producing a clean membrane fraction. The process was repeated twice. The membrane pellet was extracted in Triton X–100 as described below and the CD23 further purified by immuno-affinity chromatography. Despite the denaturing potential of ethanol, no loss of activity in these preparations could be detected when working at 4 °C. On average, the purity of the final product was around 95% as measured by gel electrophoresis (Figure 6). The yield was around 120 μg CD23 l^{-1} culture which was at least 6 fold higher than that obtained by the sucrose density gradient technique.

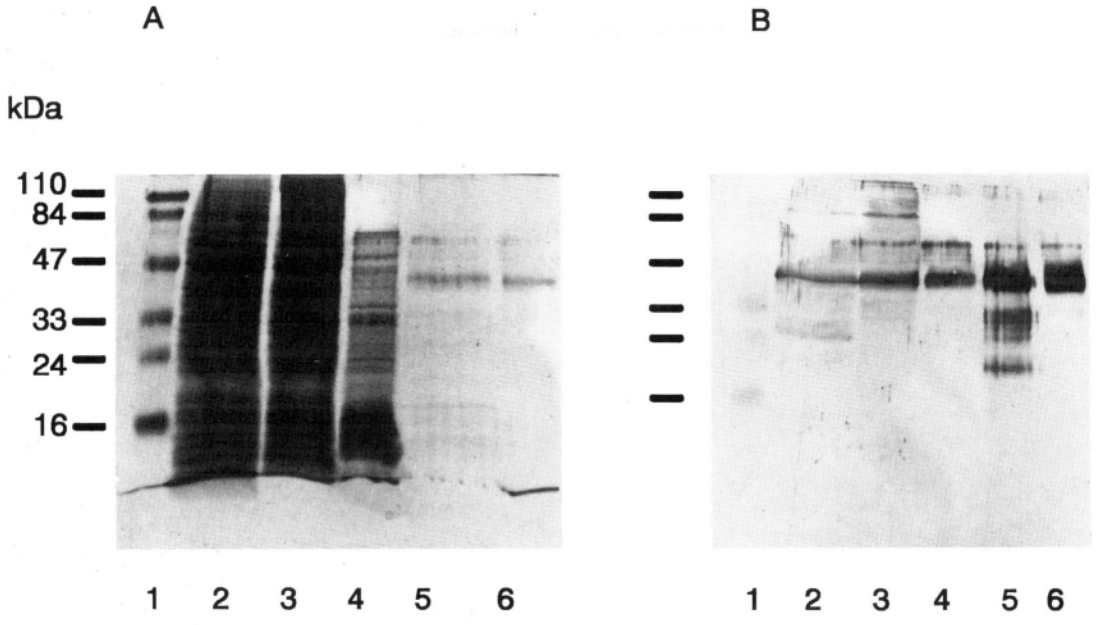

Figure 6. Gel analysis of the purification of CD23. SDS-PAGE and Western blotting analysis of full-length CD23 expressed in insect cells. Aliquots from different steps of the purification were subjected to electrophoresis on a 15% (w/v) polyacrylamide gel and stained with Coomassie blue (Panel A) or transferred onto nitrocellulose membrane and immunostained with a polyclonal antibody Rb55 (Panel B). Prestained molecular weight standards (kDa), *(Lanes 1)*, Extract of recombinant Sf9 cells *(Lanes 2)*, Membranes after ethanol precipitation *(Lanes 3)*, Detergent extract of membranes *(Lanes 4)*, MAb25 immuno-affinity elution *(Lanes 5)*, Superdex 200 final pool *(Lanes 6)*. Reproduced with permission from the Journal of Experimental Medicine, 1992, 176, pp. 389–397.

Preparation of liposomes and activity testing

The purified CD23, solubilized in 50 mM OGP, was further incorporated into fluorescent liposomes made of synthetic POPC and fluorescent DiO_{18} phospholipids solubilized in OGP (Pochon *et al.*, 1992). The detergent was then dialysed and single layer liposomes were formed which contained the transmembrane CD23. Control liposomes were made with glycophorin A protein by the same method. Recombinant CD23 incorporated into fluorescent liposomes was able to bind its ligand, CD21, on selected T and B cell lines (Figure 7). Competition of the binding with a panel of antibodies (Aubry *et al.*, 1992) proved the specificity of the interaction.

A summary of the results for each method is presented in Table 4. In conclusion, this study demonstrates that the baculovirus/insect cell protein expression system is suitable to production of native, functional transmembrane proteins. Purification of transmembrane proteins from insect cells can be achieved using essentially the same methodology as for mammalian cells. Affinity purification in detergent is a very powerful method for receptor purification. The best results were obtained starting from a membrane enriched fraction prepared by ethanol precipitation.

Part 3: Key issues for future developments

Downstream processing for the recovery of products from the baculovirus/insect cell system has been reported to be essentially similar to that involved in mammalian systems. There appear to be however several unique problems, situations and myths which arise when purifying products from this multicomponent system.

The key issue involved in cell separation and disruption as preliminary isolation steps is maintenance of compartmentalisation of the product. The literature has indicated the diversity of product localisation and therefore the need to maintain discrete boundaries during product isolation. Regimes involving complete membrane and organelle disruption are far from optimal and new protocols respecting compartmentalisation need to be developed.

Apart from cell protease release, shear sensitivity and alteration of the product during purification

Table 4. Comparison of purification methods for CD23, a transmembrane protein

Method	Number of steps	CD23 yield ($\mu g\ l^{-1}$)	Final purity (%)	Comments
Whole cell extraction	3	12	10	Impure preparation
Ethanol precipitation	5	120	90	Maximum enrichment of membranes
Sucrose density gradient	5	18	80	Poly-dispersion of membranes vesicles leading to losses
Differential centrifugation	5	7	60	Large number of centrifugation steps and low yield

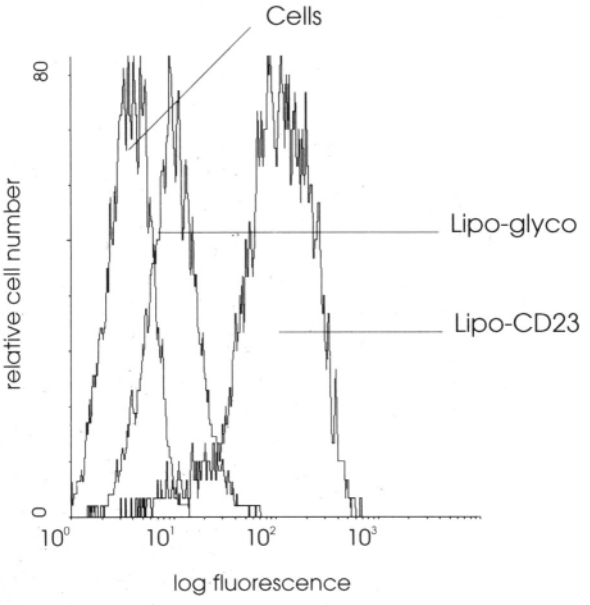

Figure 7. Biological activity of purified CD23. RPMI8226 cells (expressing the CD23 ligand, CD21, at their surface) were incubated with CD23-incorporated fluorescent liposomes (LipoCD23), glycophorin A-incorporated fluorescent liposomes (LipoGlyco) as a negative control, or without any liposomes (Cells) for 2 h at 4 °C, in presence of 2 mM Ca^{2+}. After washes, cells were analysed by flow cytometry.

should be addressed further. Additionally, the gradual retention of recombinant product over the course of hollow fibre perfusion or other cell separation protocols that involve membranes with low porosity has also been observed. There is a clear need to carefully monitor samples before and after each process to identify problem areas specific to each protein. Although knowledge of the biochemical properties of the recombinant proteins is useful for the bioreactor production process, minimisation of product loss during downstream processing requires thorough characterisation of more basic physicochemical as well as biological properties. Since prevention of cell lysis is not possible in this naturally lytic system a number of steps should be taken to avoid excessive degradation of the product once expressed. Immediate product separation is optimal.

Optimisation, for downstream processing, of the production bioreactor configuration and its mode of operation has been previously overlooked but may be an effective method to ensure maximal purification yields. During batch cultivation, the recombinant product is exposed to prolonged incubation periods which may be associated with proteolytic degradation as well as physicochemical stress including suboptimal temperature, pH and mechanical shear. In the bioreactor, the product should be separated from the cell suspension and the cell debris and then stored at low temperature. Cell separation devices incorporated into the bioreactor design can be exploited to allow direct processing of the extracellular compartment. The possibility of on-line purification processes should be considered in the future and those involving secreted products may be possible now.

Although perfusion bioreactors have the added advantage of built in cell separation and no additional microfiltration step is necessary, the production of unwanted filter cakes and molecular sieving have been observed. Experiments involving back flushing cycles and flow rate optimisation may have to be addressed depending on the product involved and the density of cells achieved in the reactor. Compared to mammalian systems, the issue of molecular sieving and membrane fouling may be a greater problem in insect cell culture due to the high densities achieved and the release of cellular components and debris due to virally induced cell lysis.

If low MOI is employed in large scale batch cultures then prior establishment of product stability at small scale is essential to determine the potential loss of product due to instability under prolonged culture con-

ditions. Low MOI protocols may only be suitable for products that are maximally expressed intracellularly prior to the onset of cell lysis. The effect of low MOI on secreted products needs to be further addressed. Another relevant disadvantage predicted to occur during low MOI cultures is the increased extent of release of intracellular proteins at the time of maximal product expression that substantially complicates downstream processing.

In conclusion the shortest possible culture time appears to be optimal for product recovery in terms of yield stability and decreased risk of contaminating proteins. The time of harvest remains one of the most important process parameters that will effect the efficiency of downstream processing. However the optimal time of harvest in terms of product expression may not correlate exactly with the optimal time of harvest in terms of product recovery.

Three main parameters must be monitored over the production process to determine the optimal time of harvest for downstream processing, i) Characterisation of cell lysis kinetics in parallel with ii) expression kinetics must be carried out before implementation of lenghthy and expessive downstream procedures. iii) Additionally the biological activity kinetics must also be monitored over the production period. This is to ensure that host cell function is maintained throughout the entire infection period allowing the continued and correct post-translational modifications, particularly around the onset of cell lysis. In most cases the optimal time of harvest in terms of product recovery does occur around the onset of cell lysis and although biological activity may be necessary at all costs, it may be possible to trade off between maximal product expression and possible protein degradation and contamination by release of host cell proteins to optimise the yield recovered.

These areas have been neglected in the literature and should be addressed as a starting point to change approaches to downstream processing.

Feed back of downstream processes to molecular biology level is also essential. Most of the developments and novel recent discoveries involving the baculovirus expression vector system have been made at the molecular biology and cloning level.

Recovery, however, may contribute 50–90% to the production costs. The need for feed back from downstream processing to genetic engineering is essential. For example, the use of early viral promoters (Chapter 9, this issue) to direct expression of the recombinant protein should be developed to solve many problems associated with late harvest times. Interdisciplinary coordination has been shown to facilitate product recovery. Fusions proteins allow efficient affinity purification with a ligand binding the fusion partner. The same purification approach is then applicable to almost any recombinant protein. Process engineering must be implemented to translate the ideas and observations at the molecular biology level into usable processes.

Scale-up of downstream processing may also be an impending hurdle. The effects of scale up on each downstream unit operation is relatively unknown, even less well documented is the collective effects of each unit operation which will inevitably complicate scale-up. As oxygen and other nutrient limitation issues have been successfully addressed at the bioreactor level extremely high density batch and continuous fermentations become reality. Each downstream unit operation will have to handle volumetrically large and concentrated process streams. The need for individual characterisation and optimisation of downstream operations will be essential. The indications are that generalisations are difficult to make about the choice of downstream processing protocols. Each step has to be assessed not only individually but collectively in order to achieve optimal recovery of the purified product.

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References

Alnemri ES, Maksymowych AB, Robertson NM & Litwack G (1991) Characterization and purification of a functional rat glucocorticoid receptor overexpressed in a baculovirus system. J. Biol. Chem. 266(6): 3925–3936.

Apelman S (1992) Separation of animal cells in continuous cell culture systems. Anim. Cell Technol. 149–154.

Aubry JP, Pochon S, Graber P, Jansen KU & Bonnefoy JY (1992) CD21 is a ligand for CD23 and regulates IgE production. Nature 358: 505–507.

Bailey JE (1995) Metabolic engineering of protein processing and signalling pathways in cultured mammalian cells. Proceedings of Cell Culture Engineering IV, San Diego USA, Cytotechnology, (in press).

Barkhem T, Carlsson B, Danielsson A, Norinder U, Frieberg H & Ohman L (1992) Production in a 100 liter stirred tank reactor of functional full length human thyroid receptor b1 in Sf–9 insect cells using a recombinant baculovirus. In Baculovirus and recombinant protein production processes, Vlak JM, Schlager EJ & Bernard AR (eds) Roche Editions, Basel, pp. 235–246.

Baylis SA, Purifoy DJM & Littler E (1991) High level expression of the Epstein Barr virus alkaline deoxyribonulease using a recombinant baculovirus: application to the diagnosis of nasopharyngeal carcinoma. Virology 181: 390–394.

Belisle B, Knoch C, Celeri C, Montgomery T, Gong T & Dougherty E (1991) Large scale in vitro production of wild type AcNPV with potential for use as a biological pesticide In Vitro 27, 3, Pt.2: 121A.

Berndt N & Cohen PTW (1990) Renaturation of protein phosphatase 1 expressed at high levels in insect cells using a baculovirus vector. Eur.J.Biochem. 190(2): 291–297.

Bordier, C (1981) Phase separation of integral membrane proteins in Triton X–114 solution. J. Biol. Chem. 256: 1604–1607.

Brandt S, Goffe RA, Kessler SB, O' Connor JL & Zale SE (1988) Membrane-based affinity technology for commercial scale purifications. Bio/Technology 6(7): 779–782.

Budde RJA, Ramdas L & Ke S (1993) Recombinant pp60csrc from baculovirus infected insect cells: purification and characterization. Prep. Biochem. 23(4): 493–515.

Cavegn C & Bernard AR (1992) A perfusion process for high density insect cell culutres. In: Baculovirus and recombinant protein production processes, Vlak JM, Schlager EJ & Bernard AR (eds) Roche Editions, Basel, pp. 262–273.

Chang C, Wang C, DeLuca HF, Ross TK & Shih CC (1992) Characterization of human androgen receptor overexpressed in the baculovirus system. Proc. Natl. Acad. Sci. USA 89(13): 5946–5950.

Chen WJ, Moomaw JF, Overton L, Kost TA & Casey PJ (1993) High level expression of mammalian protein farnesyltransferase in a baculovirus system. The purified protein contains zinc. J. Biol. Chem. 268(13): 9675–9680.

Clark R, Conroy L, Saxena A, Martin G, Apell G, Rubinfeld B, DeBoer M, Lowe D, Inlow D, Poulhazan M, Shauger A & Maiorella (1992) Expression and purification of tagged recombinant proteins from insect cells. In: Baculovirus and recombinant protein production processes, Vlak JM, Schlager EJ & Bernard AR (eds) Roche Editions, Basel, pp. 99.

Cooke R, Hale RS, Lister SG, Shah G & Weir MP (1994) The confirmation of the Sialyl Lewis X ligand changes upon binding to E selectin. Biochemistry 33: 10591–10596.

Denis D, Falgueyret JP, Riendeau D, & Abramovitz M (1991) Characterization of the activity of purified recombinant human 5 lipoxygenase in the absence and presence of leukocyte factors. J. Biol. Chem. 266(8): 5072–5079.

Domingo DL & Trowbridge IS (1988) Characterization of the human transferrin receptor produced in a baculovirus expression system. J. Biol. Chem. 263(26): 13386–13392.

Erickson JC, Finch JD & Greene DC (1994) Direct capture of recombinant proteins from animal cell culture using a fluidized bed. In Animal Cell Technology Technology, Products of today, Prospects for Tomorrow. Spier RE, Griffiths JB & Berthold W (eds) Butterworth, pp. 557–562.

Evans WH (1978) Preparation and characterisation of mammalian plasma membranes. Elsevier, Amsterdam.

Fafournoux P, Ghysdael J, Sardet C & Pouyssegur J (1991) Functional expression of the human growth factor activatable Na^+/H^+ antiporter (NHE–1) in baculovirus infected cells. Biochemistry 30(39): 9510–9515.

Findlay JBC & Evans WH (1987) Biological membranes, a practical approach. IRL Press.

Furlong AM, Thomsen DR, Marotti KR, Post LE & Sharma SK (1988) Active human tissue plasminogen activator secreted from insect cells using a baculo virus vector. Biotechnol. Appl. Biochem. 10(5): 454–464.

Giner H, Simonin F, de Murcia G & Menissier de Murcia J (1992) Overproduction and large scale purification of the human poly(ADP ribose) polymerase using a baculovirus expression system. Gene 114: 279–283.

Goodall M, Bentley TL & Jefferis R (1992) Purification of monoclonal antibody from culture supernatant produced in hollow fiber bioreactors. Anim. Cell. Technol. 621–623.

Grabenhorst E, Karger C, Reis U, Collins J, Lindnemaier W, Rollwage K, Jäger V & Conradt HS (1992) Processing of recombinant proteins: comparison of N and O glycosylation of recombinant human glycoproteins in heterologous mammalian cells and baculovirus infected Sf21 cells. In: Baculovirus and recombinant protein production processes, Vlak JM, Schlager EJ & Bernard AR (eds) Roche Editions, Basel, pp. 145–156.

Graber SG, Figler RA & Garrison JC (1992) Expression and purification of functional G protein alpha subunits using a baculovirus expression system. J. Biol. Chem. 267(2): 1271–1278.

Greenfield C, Patel G, Clark S, Jones N & Waterfield MD (1988) Expression of the human EGF receptor with ligand-stimulatable kinase activity in insect cells using a baculovirus vector. EMBO Journal 7(1): 139–146.

Gretch DG, Sturley SL, Friesen PD, Beckage NE & Attie AD (1991) Baculovirus-mediated expression of human apolipoprotein-E in Manduca sexta larvae generates particles that bind to the low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA 88(19): 8530–8533.

Gronvik KO, Frieberg H & Malstrom U (1989) Centritech Cell a new separation device for mammalian cells. Adv. Anim. Cell Biol. Technol. Bioprocesses, 428–433.

Gutowski S, Smrcka A, Nowak L, Wu DG, Simon M & Sternweis PC (1991) Antibodies to the alpha q subfamily of guanine nucleotide-binding regulatory protein alpha subunits attenuate activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by hormones. J. Biol. Chem. 266(30): 20519–20524.

Haubruck H, Mauch L, Cook NJ, Steffens U, Hunt N, Berthold H, Niemann H, Wirbelauer C & Northemann W (1993) Expression of recombinant human thyroid peroxidase by the baculovirus system and its use in ELISA screening for diagnosis of autoimmune thyroid disease. Autoimmunity. 15(4): 275–284.

Hayman AR & Cox TM (1994) Purple acid phosphatase of the human macrophage and osteoclast. Characterization, molecular properties and crystallization of the recombinant di-iron-oxo protein secreted by baculovirus-infected insect cells. J. Biol. Chem. 269(2): 1294–1300.

Hepler JR, Kozasa T, Smrcka AV, Simon MI, Rhee SG, Sternweis PC & Gilman AG (1993) Purification from *Sf*9 cells and characterisation of recombinant Gq alpha and G11 alpha. Activation of purified phospholipase C isozymes by G alpha sububunits. J. Biol. Chem. 268 (19): 14367–14375.

Herren B & Pech M (1992) A flag-tagged PDGF receptor β ectodomain expressed in *Sf*9 cells retains its high affinity binding. In: Baculovirus and recombinant protein production processes, Vlak JM, Schlager EJ & Bernard AR (eds) Roche Editions, Basel, p. 98.

Hink WF, Thomsen DR, Davidson DJ, Meyer AL & Castellino FJ (1991) Expression of three recombinant proteins using baculo virus vectors in 23 insect cell lines. Biotechnol.Prog. 7(1): 9–14.

Hodgson J (1991) Centrifugation takes a new turn. Bio/Technology 9(7): 628–629.

Hurwitz DR, Emanuel SL, Nathan MH, Sarver N, Ullrich A, Felder S, Lax I & Schlessinger J (1991) EGF induces increased ligand binding affinity and dimerization of soluble epidermal growth factor (EGF) receptor extracellular domain. J. Biol. Chem. 266(32): 22035–22043.

Jäger V, Grabenhorst E, Kobold A, Deutschmann, SM & Conradt HS (1992) High density perfusion culture of insect cells for the production of recombinant glycoproteins. In: Baculovirus and recombinant protein production processes, Vlak JM, Schlager EJ & Bernard AR (eds) Roche Editions, Basel, pp. 274–284.

Jensen RA, Beeler JF, Mohammad AH & LaRochelle WJ (1992) Characterization of baculovirus-expressed human α and β platelet-derived growth factor receptors. Biochemistry 31: 10887–10892.

Jones TL, Woodard C & Spiegel AM (1993) Baculovirus expression and purification of a soluble, mutant G-protein alpha subunit. Prot. Expr. Purif. 4(1): 64–71.

Kikuchi Y, Migita M, Takaki S, Tominaga A & Takatsu K (1994) Biochemical and functional characterization of soluble form of IL–5 receptor α **(sIL–5Rα)**. Development of EL1SA system for detection of **sIL–5Rα**. J. Immunol. Meth. 167(1–2): 289–298.

King GA, Daugulis AJ, Goosen MFA, Faulkner P & Bayly D (1989) Alginate concentration: a key factor in growth of temperature sensitive baculovirus-infected insect cells in microcapsules microencapsulation of Spodoptera frugiperda insect cell culture infected with temperature-sensitive Autographa californica nuclear polyhedrosis virus. Biotechnol. Bioeng. 34: 1085–1091.

Kompier R, Kislev N, Segal I & Kadouri A (1991) Use of a stationary bed reactor and serum free medium for the production of recombinant proteins in insect cells. Enzyme Microb. Technol. 13(10): 822–827.

Kroner KH, Krause S & Deckwer WD (1992) Cross-flow application of affinity membranes for the primary separation of proteins from crude suspensions. DECHEMA-Biotechnol.Conf. SB: 707–710.

Labrecque J, Caron M, Torossian K, Plamondon J & Dennis M (1992) Baculovirus expression of mammalian G protein alpha subunits. FEBS Letters. 304(2–3): 157–162.

Landolfi V, Zarley CD, Abramovitz AS, Figueroa N, Wu SL, & Mishkin EM (1993) Baculo virus-expressing herpes simplex virus type 2 glycoprotein D is immunogenic and protective against lethal HSV challenge. Vaccine 11(4): 407–414.

Lazarte JE, Tosi PF & Nicolau C (1992) Optimization of the production of full-length rCD4 in baculovirus infected *Sf*9 cells human CD4 gene cloning and expression in a Spodoptera frugiperda *Sf*9 insect cell culture using an Autographa californica nuclear-polyhedrosis virus vector. Biotechnol. Bioeng. 40: 214–217.

Li SL, Van PF, Paz B & Fujita-Yamaguchi Y (1992) Human insulin receptor beta-subunit transmembrane/cytoplasmic domain expressed in a baculovirus expression system: purification, characterization, and polylysine effects on the protein tyrosine kinase activity. Biochemistry 31(49): 12455–12462.

Löwenadler B, Jansson B, Paleus S, Holmgren E, Nilsson B, Moks T, Palm G, Josephson S, Philipson L & Uhlén M (1987) A gene fusion system for generating antibodies against short peptides. Gene 58: 87–97.

Lowery DE, Pasternack JM, Gonzalez-DeWhitt PA, Zurcher-Neely H, Tomich CC, Altman RA, Fairbanks MB, Heinrikson RL, Younkin SG & Greenberg BD (1991) Alzheimer's amyloid precursor protein produced by recombinant baculovirus expression. Proteolytic processing and protease inhibitory properties. J. Biol. Chem. 266(29): 19842–19850.

Luckow VA (1993) Baculovirus systems for the expression of human gene products. Curr. Opinion Biotechnol. 4: 564–572.

Luong JHT, Nguyen AL & Male KB (1987) Affinity cross-flow filtration for purifying biomolecules. Bio/Technology 5: 564–566.

Maeda S & Kawai T (1985) Production of human a interferon in silkworm using a baculovirus vector. Nature 315: 592–594.

Maiorella B, Dorin G, Canon A, Harano D (1991) Crossflow microfiltration of animal cells. Biotechnol. Bioeng. 37: 121–26.

Mawson AJ (1993) Cross flow microfiltration of cell suspensions. Australas.Biotechnol. 3(6): 348–352.

McGlynn E, Becker M, Mett H, Reutener S, Cozens R & Lydon NB (1992) Large-scale purification and characterisation of a recombinant epidermal growth-factor receptor protein-tyrosine kinase. Modulation of activity by multiple factors. Eur. J. of Biochem. 207(1): 265–275.

Medin JA, Hunt L, Gathy K, Evans RK & Coleman MS (1990) Efficient, low cost protein factories: expression of human adenosine deaminase in baculovirus infected insect larvae. Proc. Natl. Acad. Sci. USA. 87: 2760–2764.

Menge U, Fraune E, Lehmann J & Kula MR (1987) Purification of proteins from cell culture supernatants. Dev. Biol. Stand. 66: 391–401.

Mills A, Allet B, Bernard A, Chabert C, Brandt E, Cavegn C, Chollet A & Kawashima E (1993) Expression and characterization of human D4 dopamine receptors in baculovirus-infected insect cells. FEBS Letters 320(2): 130–134.

Miyajima A, Schreurs J, Otsu K, Kondo A, Arai K & Maeda S (1987) Use of the silkworm, Bombyx mori and an insect baculovirus vector for high level expression and secretion of biologically active mouse interleukin 3. Gene 58: 273–278.

Morishita K & Maeda S (1991) Characterization and purification of the biologically active v-sis protein generated in silkworm larvae within the Bombyx mori nuclear polyhedrosis virus vector system J.Biotechnol. 20(3): 223–234.

Murriammer DW & Goochee CF (1988) Scale-up of insect cell cultures: protective effects of Pluronic F–68. Bio/Technology 6(12): 1411–1418.

Nachman M, Azad ARM & Bailon P(1992) Membrane-based receptor affinity chromatography. J. Chromatogr. 597: 155–66.

Nakamura N, Sudo T, Sakurai T, Matsuda S & Yanai A (1992) Molecular cloning of feline interferon cDNA by direct expression and its mass production using silkworm. Biochem. Eng. 2001. 31–34.

Narum DL, Welling GW & Thomas AW (1993) Ion exchange immunoaffinity purification of a recombinant baculovirus Plasmodium falciparum apical membrane antigen, PF83/AMA 1. J. Chromatogr. 657(2): 357–363.

Nutt EM, Jain D, Lenny AB, Schaffer L, Siegl PK & Dunwiddie CT (1991) Purification and characterization of recombinant antistasin: a leech-derived inhibitor of coagulation factor Xa. Arch. Biochem. Biophys. 285(1): 37–44.

O'Reilly DR, Miller LK & Luckow VA (1992) Baculovirus expression vectors a laboratory manual. Oxford University Press.

Overton L & Kost TA (1995) Potential application of insect cell-based expression systems in the bio/pharmaceutical industry. In: Baculovirus expression systems and biopesticides. Wiley-Liss Publishers, pp. 233–242.

Park S, Marshall MS, Gibbs JB & Jove R (1992) Reconstitution of interactions between the Src tyrosine kinases and Ras GTPase-activating protein using a baculovirus expression system. J. Biol. Chem. 267(16): 11612–11618.

Parker EM, Kameyama K, Higashijima T & Ross EM (1991) Reconstitutively active G protein coupled receptors purified from baculovirus infected insect cells. J. Biol. Chem. 266(1): 519–527.

Paul JI, Tavare J, Denton RM & Steiner DF (1990) Baculovirus-directed expression of the human insulin receptor and an insulin-binding ectodomain J. Biol. Chem. 265(22): 13074–13083.

Peng S, Sommerfelt M, Logan J, Huang Z, Jilling T, Kirk K, Hunter E & Sorscher E (1993) One-step affinity isolation of recombinant

protein using the baculovirus/insect cell expression system. Prot. Expr. Purif. 4(2): 95–100.

Pochon S, Graber P, Yeager M, Jansen K. Bernard AR, Aubry JP & Bonnefoy JY (1992) Demonstration of a second ligand for the low affinity receptor for immunoglobulin E (CD23) using recombinant CD23 reconstituted into fluorescent liposomes. J. Exp. Med. 176: 389–397.

Possee RD (1986) Cell surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. Virus Res. 5: 43–59.

Prehaud C, Harris RD, Fulop V, Koh CL, Wong J, Flamand A & Bishop DHL (1990) Expression, characterization and purification of a phosphorylated rabies nucleoprotein synthesised in insect cells by baculovirus vectors. Virology 178: 486–497.

Prior CP, Doyle KR, Duffy SA, Hope JA, Moellering BJ & Prior GM (1989) The recovery of highly purified biopharmaceuticals from perfusion cell culture bioreactors J.Parenter.Sci.Technol. 43(1): 15–23.

Quelle FW, Caslake LF, Burkert RE & Wojchowski DM (1989) High-level expression and purification of a recombinant human erythropoietin produced using a baculovirus vector. Blood. 74(2): 652–627.

Rankl NB, Rice JW, Gurganus TM, Barbee JL & Burns DJ (1994) The production of active protein kinase C δ in insect cells is greatly enhanced by the use of the basic protein promoter. Prot. Expr. Purif. 5: 346–356.

Reif OW, Nier V, Bahr U & Freitag R (1994) Immobilized metal affinity membrane adsorbers as stationary phases for metal interaction protein separation. J. Chromatogr. 664(1): 13–25.

Reiländer H, Boege F, Vasudevan S, Maul G, Hekman M, Dees C, Hampe, W, Helmreich EJ & Michel H. Purification and functional characterization of the human beta 2 adrenergic receptor produced in baculovirus infected insect cells. FEBS Letters. 282(2): 441–444.

Reuveny S, Kim YJ, Kemp CW & Shiloach J (1993a) Production of recombinant proteins in high density insect cell cultures. Biotechnol. Bioeng. 42(2): 235–239.

Reuveny S, Kim YJ, Kemp CW & Shiloach J (1993b) Effect of temperature and oxygen on cell growth and recombinant protein production in insect cell cultures Appl. Microbiol. Biotechnol. 38(5): 619–622.

Riese U, Lutkemeyer D, Heidemann R, Büntenmeyer H & Lehmann J (1994) Re-use of spent cell culture medium in pilot scale and rapid preparative purification with membrane chromatography. J. Biotechnol. 34: 247–257.

Robishaw JD, Kalman VK & Proulx KL (1992) Production, processing and partial purification of functional G protein beta gamma subunits in baculovirus-infected insect cells. Biochem. J. 286 (Pt 3): 677–680.

Schlaeger EJ, Loetscher H & Gentz R (1992) Fermentation scale-up: production of soluble human TNF receptors. In: Baculovirus and recombinant protein production processes, Vlak JM, Schlager EJ & Bernard AR (eds) Roche Editions, Basel, pp. 201–208.

Schmidt C, Berglof JH & Lindquist LO (1994) Impact of improved chromatographic media on productivity and process design in downstream processing. In: Animal Cell Technology Technology, Products of today, Prospects for Tomorrow. Spier RE, Griffiths JB & Berthold W (eds) Butterworth, pp. 553–556.

Sheeler, P (1981) Centrifugation in Biology and Medecine, John Wiley, New York.

Shiloach J, Kaufman JB & Kelly RM (1986) Hollow fiber microfiltration methods for recovery of rat basophilic leukemia cells (RBL 2H3) from tissue culture media. Biotechnol.Prog. 2(4): 230–233.

Singer WD, Miller RT & Sternweis PC (1994) Purification and characterization of the alpha subunit of G13. J. Biol. Chem. 269(31): 19796–19802.

Smrcka AV, Hepler JR, Brown KO & Sternweis PC (1991) Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. Science. 251(4995): 804–807.

Springer TA & Lasky LA (1991) Sticky sugars for selectins. Nature 346: 196–197.

Stauffer KA, Kumar NM, Gilula NB, & Unwin N (1991) Isolation and purification of gap junction channels. J. Cell Biology 115(1): 141–150.

Strathmann M & Simon MI (1990) G protein diversity: a distinct class of alpha subunits is present in vertebrates and invertebrates. Proc. Natl. Acad. Sci. USA. 87(23): 9113–9117.

Summers MD & Smith GE (1987) A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Experimental Station Bulletin 1555, Texas A&M, College Station.

Tang WJ, Kupinski J & Gilman AC (1991) Expression and characterization of calmodulin activated (Type I) adenylylcyclase. J. Biol. Chem. 266: 8595–8603.

Taylor SJ, Chae HZ, Rhee SO & Exton JH (1991) Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. Nature. 350(6318): 516–518.

Tokashiki M & Takamatsu H (1993) Perfusion culture apparatus for suspended mammalian cells. Application in monoclonal antibody and therapeutic and diagnostic protein production. Cytotechnology 13(3): 149–159.

Tolbert WR & Prior CP (1988) Recovery of highly purified biopharmaceutical proteins from mammalian perfusion culture systems. In World-Biotech-Rep., On Line publications, London, pp. 179–184.

Ueda N, Iniguez-Lluhi JA, Lee E, Smrcka AV, Robishaw JD & Gilman AG (1994) Gprotein beta gamma subunits. Simplified purification and properties of novel isoforms. J. Biol. Chem. 269(6): 4388–4395.

Van-Reis R, Leonard LC, Hsu CC & Builder SE (1991) Industrial scale harvest of proteins from mammalian cell culture by tangential flow filtration. Biotechnol. Bioeng. 38(4): 413–122.

Vihko P, Kurkela R, Porvari K, Herrala A, Lindfors A, Lindqvist Y & Schneider G (1993) Rat acid phosphatase: overexpression of active, secreted enzyme by recombinant baculovirus-infected insect cells, molecular properties and crystallization. Proc. Natl. Acad. Sci. USA. 90(3): 799–803.

Vissavajjhala P & Ross AH (1990) Purification and characterization of the recombinant extracellular domain of human nerve growth factor receptor expressed in a baculovirus system. J. Biol. Chem. 265(8): 4746–4752.

Wang MY, Bentley WE & Vakharia V (1994) Purification of a recombinant protein produced in a baculovirus expression system by immobilized metal affinity chromatography. Biotechnol. Bioeng. (1994) 43(5): 349–356.

Webb NR, Madoulet C, Tosi PF, Broussard DR, Sneed L, Nicolau C. & Summers MD (1989) Cell surface expression and purification of human CD4 produced in baculovirus infected insect cells. Proc. Natl. Acad. Sci. USA 86(20): 7731–7735.

Wells DE & Compans RW (1990) Expression and characterization of a functional human immunodeficiency virus envelope glycoprotein in insect cells. Virology 176: 575–586.

Wickham TJ, Davis T, Granados RR, Hammer DA, Shuler ML & Wood HA (1991) Baculo virus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection Biotechnol. Lett. 13(7): 483–488.

Wingfield PT, Graber P, Buell G, Rose K, Simona MG & Burleigh BD (1987) Preparation and characterization of bovine growth hormones produced in recombinant *Escherichia coli.* Biochem. J. 243: 829–839.

York JD, Chen ZW, Ponder JW, Chauhan AK, Mathews FS & Majerus PW (1994) Crystallization and initial X-ray crystallographic characterization of recombinant bovine inositol polyphosphate 1-phosphatase produced in *Spodoptera frugiperda* cells. J. Mol. Biol. 236(2): 584–589.

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PART IV
APPLICATIONS

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Parvovirus diagnostics and vaccine production in insect cells

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Key words: insect cells, parvoviruses, virion, immune response, capsid proteins, VLPs

Introduction

Baculoviruses have been successfully used to express large amounts of heterologous proteins for various applications (Luckow & Summers, 1988; Luckow, 1991). Recombinant baculoviruses can be produced on a large scale in insect cells. Insect cell cultures offer an attractive alternative to traditional systems for manufacturing of pharmaceuticals, biologicals and diagnostics products.

High level expression of heterologous genes makes the baculovirus expression system specially suitable for those cases where large amounts of protein are needed, for instance, production of subunit vaccines or diagnostic reagents. These new generation vaccines will help to obviate the risks associated to the production and use of live-modified viruses in classical vaccines. The aim of this review is to summarize the use of recombinant baculoviruses for the production of vaccines and diagnostic reagents useful for parvoviruses (Table 1). Also, possible ways to improve the levels and the quality of the expression are presented. Finally, an study about the genetic stability of the system is shown.

Parvoviruses

The family *Parvoviridae* is composed by a group of small, non enveloped, linear single-stranded DNA viruses (for a review, see Cotmore & Tattersall, 1987). They depend on certain cellular helper functions. Therefore, virus replication will require rapidly dividing tissues, such as fetal tissues, intestinal epithelium and the hemopoietic system.

Parvoviruses cause a number of diseases in man and animals. They are endemic and widely prevalent in populations of their natural hosts, between 70–90% of world populations are seropositive for the respective parvoviruses. In this review, particular emphasis is given to some of the most important parvoviruses, v.g, the human parvovirus B19 and two economically relevant veterinary viruses: porcine parvovirus (PPV) and canine parvovirus (CPV). B19 is distantly related to the other autonomous parvoviruses. In contrast, PPV and CPV share many structural characteristics (Tsao *et al*., 1991) and are classified in the rat parvovirus group.

B19 parvovirus has been associated with a variety of different human diseases including aplastic crisis, erythema infectiosum, hydrops fetalis and polyarthralgia syndrome in normal adults. PPV is one of the major causes of reproductive failure in pigs. The infection occurs without clinical symptoms in adults, however the virus can cross the placental barrier during the infection and cause the death of the foetuses or embryos. CPV is responsible for acute enteritis in young dogs, that may cause the death of the animal in some specially serious cases. CPV is antigenically and genetically very related (98% homology) with other relevant parvoviruses as feline panleukopenia virus and mink enteritis virus. Therefore, vaccines used to induce protection in dogs are predicted to have the same effect in other hosts, as cats and minks.

Parvovirus infections are mainly controlled by the antibody response. A number of classical vaccines based on inactivated or attenuated viruses are available for controlling some of the most relevant diseases induced by parvoviruses. In general, since the parvoviruses do not replicate well *in vitro*, the possibility of developing a recombinant vaccine was very inter-

esting. This is specially true for the case of B19, where no large scale culture system is still available.

The virion. Genetic strategy

Non enveloped icosahedral capsids of autonomous parvoviruses have a diameter of approximately 25 nm and are composed by 60 capsomeres. Two types of capsids can be found in nature. Full capsids or capsids containing DNA show a density in CsCl gradients of 1.38 g/cm^3. Empty capsids, without DNA, are also abundantly produced and they have a density of 1.33 g/cm^3.

The viral genome is formed by a single-stranded linear DNA molecule of approximately 5000 bases. The genetic strategy and genomic organization are similar in all the parvoviruses. There are two large open reading frames (ORF) that cover the length of the viral genome. The left ORF codes for the non structural proteins. The capsid proteins are encoded in the right ORF and they are forming a nested structure. There are two structural proteins, the minor structural protein or VP1 (81–83 kDa) and the major structural protein or VP2 (58–67 kDa). VP1 and VP2 are different splicing products from the same gene. VP2 is the amino terminal truncated form of VP1. In native virions, the capsid contains 5–10% of VP1 and 90–95% VP2. In the infectious particles, some VP2 is proteolitically degraded to yield VP3.

Immune response to parvoviruses

Parvoviruses elicit high liters of antibodies, usually the humoral response is enough to control the disease. Parvovirus infection can be controlled by passive transfer of antibodies to the affected individuals. Thus, the cell mediated immunity does not play a major role, if any, in the control of the virus. To analyze the specificity of the antibody response, protein fragments were expressed using a procaryotic system. When these fragments were probed with a collection of neutralizing antibodies, all of them recognized VP2-derived fragments (Lopez de Turiso *et al.*, 1991). Same results were obtained using PEPSCAN technology (Langeveld *et al.*, 1993). Consequently, in CPV and PPV, VP2 was the target for the expression experiments.

In B19 the situation is different, the 3D structure shows major differences between this virus and the family of the rat parvoviruses (Agbandje *et al.*, 1994).

Table 1. Expression of parvovirus proteins in insect cells

Parvovirus	Protein	References
AAV-2	VP1, VP2	Ruffing *et al.*, 1992
ADV	VP1, VP2	Christensen *et al.*, 1993
B19	VP1, VP2	Kajigaya *et al.*, 1991; Brown *et al.*, 1992
MVM	VP2	Tullis *et al.*, 1991
CPV	VP2	López de Turiso *et al.*, 1992; Saliki *et al.*, 1992
MEV	VP2	Christensen *et al.*, 1994
PPV	VP2	Martinez *et al.*, 1992

Abbreviations: AAV-2: Adeno-associated virus type 2, ADV: Aleutian disease virus, MVM: minute virus of mice, PPV: Procine parvovirus, CPV: Canine parvovirus, MEV: Mink enteritis virus.

The antigenic properties seem different, specially in the role of VP1 unique sequences in virus neutralization. As in PPV and CPV, VP2 was the major target of neutralization (Sato *et al.*, 1991). The amino terminus of VP2 was able to elicit neutralizing antibodies. However, also the amino terminal portion of the unique region of the VP1 protein and the VP1-VP2 junction regions contain neutralizing domains (Saikawa *et al.*, 1993). The unique region of VP1, which contains several neutralizing epitopes, has been localized to the exterior of the virus (Rosenfeld *et al.,* 1992). This result will have a deep influence on ulterior results.

Expression of capsid proteins in insect cells

First attempts of expression of parvovirus VP2 genes were carried out in *E. coli* using different systems (Sisk and Berman, 1987; Lopez de Turiso *et al.*, 1991). The expression products were consistently recognized by sera of infected individuals. However, the immunogenicity of these products was very poor since the proteins were produced in a denatured state, which made them totally insoluble and not useful for vaccine production.

To overcome this problem, the parvovirus structural genes were expressed in insect cells using the baculovirus system. The expression was firstly based on the use of the polyhedrin promoter. In B19, and since splicing in AcNPV-infected Sf9 cells is inefficient, two different recombinants carrying the genes for VP1 and VP2 were prepared and used to coinfeet insect cells (Brown *et al.*, 1991; Kajigaya *et al.*,

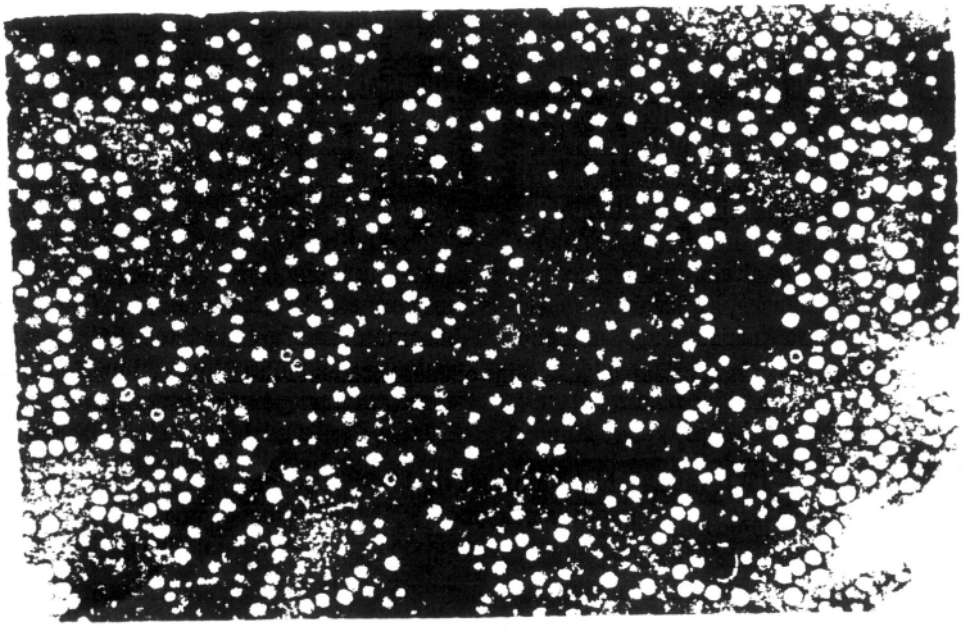

Figure 1. Electron micrograph of PPV VP2 particles.

1991). Empty capsids containing both B19 VP1 and VP2 were produced. A dual recombinant baculovirus expressing simultaneously VP1 and VP2 was also prepared (Brown *et al*., 1991). The empty capsids contained fixed amounts of VP1 and VP2 in a similar stoichiometry to the original virions. The ratio of VP1 to VP2 in empty capsids could be increased from the normal amount of about 5 to 40% by manipulation of the ratio of the coinfecting B19 VP1 and VP2-expressing baculoviruses. VP1-enriched capsids were particularly potent in eliciting neutralizing antibodies (Rosenfeld *et al*., 1992).

Surprisingly, recombinant baculoviruses expressing only the VP2 gene were also able to produce empty capsids in B19, PPV and CPV (Brown *et al*., 1991, Kajigaya *et al*., 1991, Martinez *et al*., 1992, Lopez de Turiso *et al*., 1992, Saliki *et al*., 1992). The recombinant VP2 was identical in size to the viral and it was recognized by specific antisera against parvoviruses. Moreover, the recombinant protein was able to hemagglutinate red blood cells in a similar extent to the original virions. This property was a consequence of the ability of these recombinant proteins to assemble "in vitro", forming virus-like particles (VLPs) similar in size and morphology to the original virions (Fig. 1). Altogether, these characteristics were already suggesting that the immunological properties of these VP2 particles would be very similar to those of the natural viruses. In fact, a topographical analysis of the recombinant capsids and CPV virions using gold-labeled MAbs and immunoelectron microscopy indicated a similar topology for both types of capsids (Cortés *et al*., 1993).

Optimizing the expression of VLPs

Because of the commercial relevance of these products, several strategies have been applied to improve the levels of VLPs expression. However, the application of these methods to these proteins not always yielded the expected results. The case of PPV VP2 will be discussed as a model, since several vectors and different constructs were prepared. Initially, PPV VP2 gene was cloned (Martinez *et al*., 1992) under the control of the polyhedrin promoter in pJVP10z (Vialard *et al*., 1990), which carries the lac Z gene as reporter under the control of the p10 promoter. In this case the levels of expression were around 5–10 $\mu g/10^6$ cells. Subcloning in other polyhedrin-based transfer vectors as pAcDZ1 (Zuidema *et al*., 1990) or pAcYM1 (Matsuura *et al*., 1987) did not improve the levels of expression, despite they have different sequences around the polyhedrin ATG start codon, including or not portions of the coding sequence of the polyhedrin gene (Casal *et al*., 1992). The level of VP2 expression under the con-

Table 2.

pAcAs3				pJVP10z			
Exp. #	Total[a] Protein	HA	HA/μg	Exp. #	Total Protein	HA	HA/μg
1	2.0	1/640,000	320	1	12.2	1/640,000	52.4
2	1.2	1/640,000	533	2	6.2	1/640,000	103
3	1.0	1/320,000	320	3	5.8	1/640,000	133

[a] Total protein in mg.

trol of the polyhedrin promoter was similar in every case and independent of the competition with other promoters, as p10, in the same transfer vectors. The presence or absence of extra sequences, up to 85 bp, at the 5'end of the gene was also irrelevant (Casal *et al*., 1992).

However, the change of the promoter used for the expression was more relevant, as it was shown by using p10-based transfer vectors. PPV VP2 gene was subcloned in the vector pAcAs3 (Vlak *et al*., 1990), under the control of the p10 promoter, which also carries the β-galactosidase as reporter, but under the control of the weak *hsp*20 promoter. Although the levels of expression were similar, the quality of these new capsids produced by the vector pAcAs3 was superior (Table 2), because: 1. – The ratio of VLPs/μg of total protein, that give. us an estimation of the purity of the capsids was between 3-5 times better for the construction with the p10 promoter. 2. - The presence of β-galactosidase was very low and could be removed after a single step of purification. 3. – When the VLPs were analyzed by ultracentrifugation in sucrose gradients, a sharp peak was observed in the sucrose profile, in contrast to previous preparations with polyhedrin-derived capsids, where usually the VLPs peak was contaminated by a shoulder due probably to the presence of monomers of VP2. Finally, 4. – the preparations at the electron microscope look rather homogeneous in comparison to those coming from pJVP10Z. Besides, there are other advantages of the p10 promoter, for instance, it can be used to infect larvae since it maintains the polyhedrin gene.

Use of parvovirus VLPs in vaccination

The availability of unlimited supplies of VLPs has allowed the development of new diagnostic tests and vaccines based on these non replicating agents, produced in a mammalian-free system, so recombination or encapsidation of pathogenic genes or agents is very unlikely. These vaccines will be extremely safe and innocuous for the vaccinee. Several examples of the efficacy of these VLPs to confer protection are discussed below.

Since PPV does not cause any relevant pathogeny in adult animals, the only way to demonstrate the efficacy of these VP2 particles as potential vaccines was the vaccination of pregnant sows or gilts in order to study its effect on the litter. Fig. 2 shows the results of an experiment of vaccination of gilts with recombinant PPV/VP2 capsids. Two gilts were vaccinated with two doses of 3 μg of recombinant capsids. It is remarkable the minimal amount of antigen used in this experiment (3 μg/dose). The gilts were vaccinated with three weeks interval, then they were artificially inseminated and about two months later they were challenged with a high dose of virulent virus. A control gilt was challenged simultaneously. Twenty days later, the gilts were sacrificed and the foetuses surgically extracted to check for clinical manifestations of disease. Foetuses coming from vaccinated gilts were all alive and clinically healthy. However, 5 out of 9 foetuses from the non-vaccinated gilt were dead. Three of the remaining four showed clear manifestations of the disease which include large edemas, pulmonary stasis and pronounced tissue destruction. This experiment, together with others performed later, with more pigs and similar results, clearly indicate the capability of these recombinant capsids to elicit full protection of the vaccinated animals against the disease.

In dogs, the major target of the canine parvovirus disease are young animals (<1 year old). The experimental conditions of the vaccination were different to PPV but the results were identical regarding full protection (Lopez de Turiso *et al*., 1992). In this case, a collection of 14 weeks-old Beagle dogs were used in the immunization experiments. Different doses of

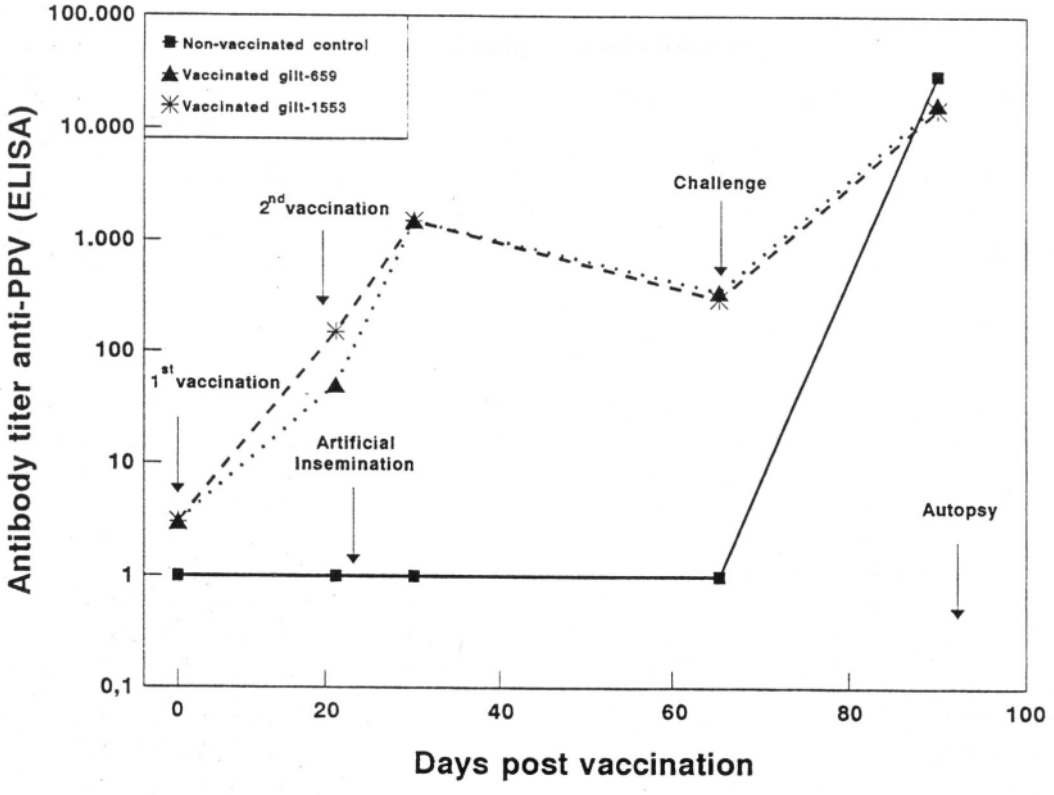

Fig. 2. Course of antibody production in gilts vaccinated with PPV VP2 particles. The results are given for the three gilts of the first experiment. An ELISA was used for the detection of anti-PPV antibodies. Titers were taken at those serum dilutions which yielded absorption values three times the blank.

CPV VP2 capsids were tested as shown in Fig. 3. The use of only 1 μg of capsids per dog elicited inhibition of hemagglutination titers in sera around 1/2000 (it is generally accepted that liters around 1/80 are enough to protect the animals). Given these high antibody liters, when the animals were challenged with a virulent CPV virus isolated from the feces of an infected dog, neither viremia or new antibody production were detected and a full protection of the animals was obtained (Fig. 3). As mentioned above, the high sequence homology between CPV and other parvoviruses as FPLV and MEV, suggested that this vaccine could also protect minks or cats. This theory was tested in minks and turned out to be the case. One single dose of 3 μg of CPV VP2 capsids was effective to confer total protection to the immunized minks and prevent virus secretion (Langeveld *et al.*, 1995). This low amount of antigen is cost-effective and applicable on large-scale vaccination programs.

In human parvovirus B19, only experiments in animal models have been reported. The capsids are able to induce high antibody titers even in the absence of adjuvant (Brown *et al.*, 1994). However, small variations in the protein composition of the capsid have a large influence on the immune response of inoculated animals. Rabbits injected with VP2 particles failed to induce antibodies able to neutralize virus infectivity. In contrast, animals injected with VP1 plus VP2 capsids make neutralizing antisera comparable in titer to convalescent-phase human sera (Kajigaya *et al.*, 1991). Then, it has been shown that the unique region of VP1 is external to the capsid, as the dominant antibody species in normal immune serum are directed against VP1 (Kurtzman *et al.*, 1989). Changing the VP1 content of the recombinant capsids, a correlation was observed between the VP1 content and the potency of virus neutralizing antibodies (Bansal *et al.*, 1993). The maximum effect was seen with a VP1 content of 25%, however this effect may be dependent of the animals and adjuvant used. The use of the double recombinant baculovirus expressing fixed amounts of VP1 and VP2 could be specially advantageous for

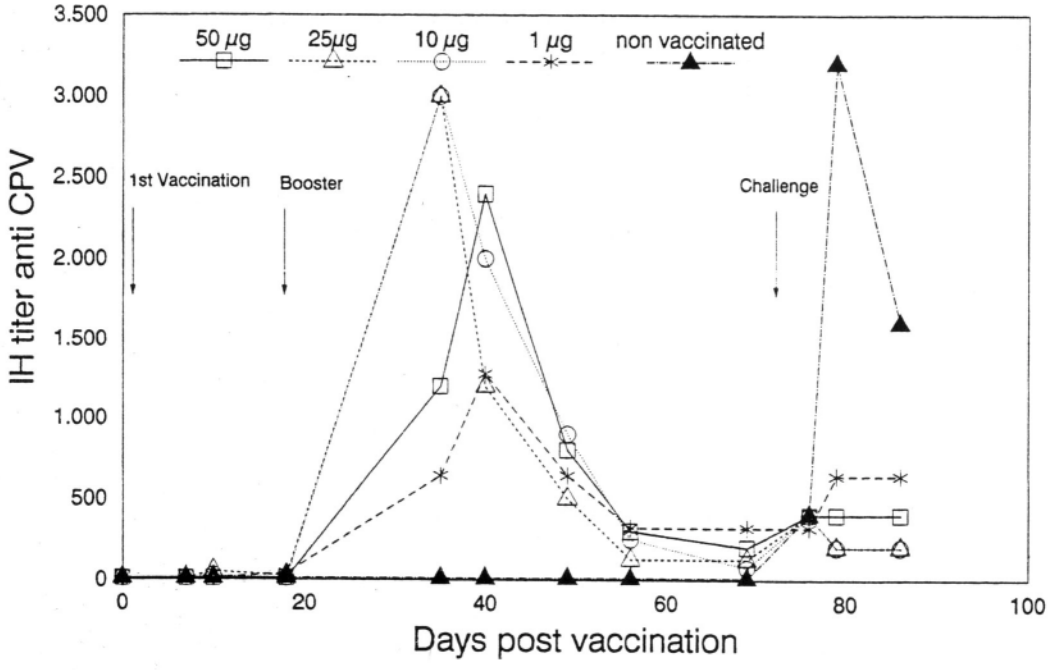

Fig. 3. Course of antibody production in dogs vaccinated with CPV VP2 particles. The antibody production was evaluated by hemagglutination inhibition. The different doses used in the experiment are showed in the figure. Each value represents the average for two different dogs.

the development of a vaccine in B19, since it avoids the problems associated with the coinfection of insect cells with different baculoviruses. Further research will be needed on these B19 capsids before application on vaccination programs.

Use of VLPs in diagnosis

The diagnosis of parvoviruses is a practical application with important repercussion in the field of vaccines, specially in those cases, as PPV and CPV, where massive vaccination programs are in progress and the animals receive several doses during their lifetime. These recombinant capsids can be used in different techniques such as indirect or competition ELISA for the determination of the serological status of a population (v.g. pig farms in order to evaluate the necessity of a revaccination of the gilts or the presence of an outbreak of PPV).

For PPV, traditional diagnostic techniques were based on indirect immunofluorescence assays (IFA) and/or hemagglutination inhibition (Joo *et* al., 1976). These techniques are difficult to automatize for processing a large number of samples. To set up an ELISA procedure, the ability of the three available types of PPV capsids for being used as antigen was compared, v.g. native virus and recombinant capsids derived from the polyhedrin and p10 promoter expression. Fig. 4 shows the titration of the different antigens in an indirect ELISA, using standard positive and negative PPV antisera as a reference. Recombinant capsids from pAcAs3 perform equal if not better than the original virions and they can be used up to 0.12 µg/well, indicating that five times less capsids from p10 origin are necessary as coating antigen. Nevertheless, the results obtained with the three types of capsids and different pig sera were identical, regarding specifity, in this ELISA. The indirect ELISA allows the quantification of the anti-PPV antibody titer in pig sera. This titer gives an indication about the health status of the reproductive animals and the necessity of carrying out a revaccination or not. Also, a blocking ELISA based on the competition for binding to PPV VLPs between the serum sample and a PPV-specific MAb has been developed. This blocking assay allows to determine if a sample is positive or not in a single step. Kits based on these assays are being currently commercialized. The same techniques described for PPV were applied to CPV with similar results.

B19 capsids have been used for the development of diagnostic assays such as IFA (Brown *et al*., 1990) and

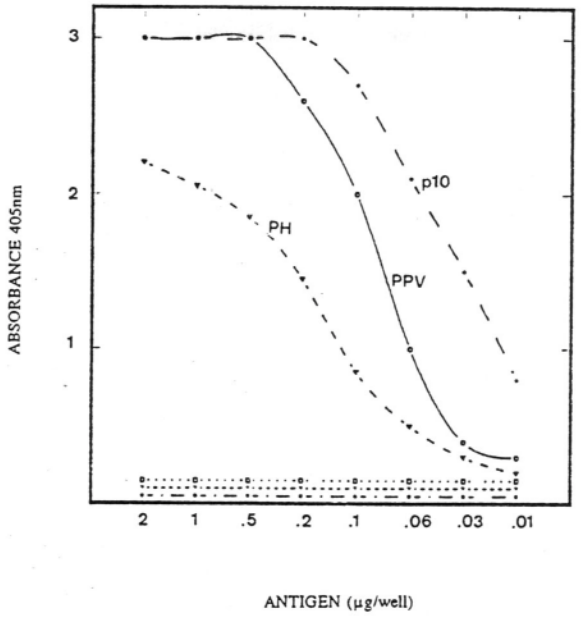

Fig. 4. Use of PPV VP2 particles in an indirect ELISA. Three different capsids were compared as coating antigen. PPV: viral particles. p10: p10 promoter-derived capsids. PH: polyhedrin-based capsids. They were tested with two reference sera (positive and negative) according to standard methods.

ELISA assays (Salimans *et al*., 1992). No such assays were available before. The IFA based on whole insect cells expressing VP1 and the ELISA based on VP2 or VP1/2 capsids were shown to correlate with a radioimmunoassay based on native virus for the detection of anti-B19 IgM and IgG. The use of both techniques, IFA and ELISA, has allowed to determine the avidity of IgG antibodies to distinguish recent infections from preexisting immunity (Gray *et al*., 1993). Other possible application is to distinguish acute phase (< 1 week after viremia) and convalescent sera (weeks to months after infection) based on the different ability to recognize linear determinants unique to VP1 or VP2 protein. Acute phase sera predominantly recognize linear epitopes specific to the VP2 protein with convalescent sera recognizing mainly VP1-specific linear epitopes (Schwartz *et al*., 1988; Kurtzman *et al*., 1989). These new B19 tests present considerable advantages over existing tests based on native virus in that they do not need virus isolates difficult and expensive to obtain and do not require the use of radio isotopes. Both the VP1 - IFA and the ELISA based on the VP2 capsids have been developed and marketed by diverse companies.

Effect of virus passage on the genetic stability and VLPs production

One of the major concerns relative to the use of recombinant baculoviruses is the generation of defective AcNPV genomes by serial passages in cell culture. In fact, using continuous infection of Sf21 cells with a recombinant baculovirus in a bioreactor, Kool *et al*., (1991) demonstrated the generation of defective interfering particles, in which the genome had a deletion from 1.7 to 45 map units. To study the potential generation of mutants in the recombinant baculoviruses expressing PPV and to study the effect on the production of the recombinant product, 20 consecutive undiluted passages of the recombinant viruses were done on Sf9 cells. Extracellular virus was used as the starting material for the isolation of DNA at passages 0, 10 and 20. Purified DNA was subsequently digested with restriction endonucleases *Eco*RI and *Bam*HI. No alterations in the restriction pattern of the enzymatic digestions was observed in the first 10 passages, although two bands on the *Eco*RI pattern were weaker after 20 passages. The presence of the VP2 gene at the different passages was checked by Southern blot experiments. No effect was observed in the relative amounts of the VP2 gene due to virus passage. In con-

Table 3. Effect of virus passage on PPV VLPs production

Passage	Viral Titer	Total[a] Protein	HA	HA/μg
0	2.8×10^7	12.2	1/640,000	52.4
10	3.2×10^7	8.7	1/320,000	36.7
20	3.9×10^7	4.7	1/160,000	34.0

[a] In mg.

clusion, defective genomes started to appear at about 20 passages although without affecting, apparently, the presence of the VP2 gene in the genome of the recombinant viruses.

The effect of virus passage on the virus liter and PPV VLPs expression was also analyzed. The results are shown in Table 3. Little effect of the virus passage on the viral titer of the resulting baculovirus was observed. However, the total protein production in the case of PPV suffered a considerable decrease in function of the virus passage, as it was also observed in the HA titer (in units/ml). The ratio of HA units/μg of protein, that gives a reasonable estimation of the presence of VLPs in the purified VP2 preparations, showed a decrease, higher in the first 10 passages. This result shows again the relevance of keeping at a minimum the number of passages of the recombinant baculoviruses used for production purposes.

Future applications of parvovirus VLPs

Parvovirus VLPs have been proposed as vehicles for gene transfer and for the development of polyvalent vaccines. Parvovirus VLPs are being used in the development of methods for gene transfer into tissue culture cells, using the endosomolytic properties of viruses and capsids. The tropism of parvovirus B19 for cells of the erythroid lineage may allow the targeting of genes for the repair of disorders specific to this cell lineage (Brown, 1994).

Other potential application for parvo VLPs is their use as carrier vaccines. Recently, it has been described the use of chimeric parvovirus B19 capsids for the presentation of foreign epitopes (Brown *et al*., 1994) and as platforms for the delivery of other proteins (Miyamura *et al*., 1994). Epitopes from mouse hepatitis virus (MHV) and herpes simplex virus 1 (HSV–1) were inserted at the N-terminus and at a loop of the surface of the B19 capsid. Resulting capsids were immunogenic in mice, which were partially protected against a challenge infection with either MHV or HSV.

In a recent study (Sedlik *et al*., 1995), sequences corresponding to T and B cell poliovirus epitopes have been inserted at the N-terminus of PPV VP2 particles. The chimeric PPV capsids containing poliovirus C3:T epitope induced an specific T cell response *in vitro* and *in vivo*. In contrast, hybrid particles containing the poliovirus C3:B epitope in the N-terminus of VP2 did not elicit any antibody response. This difference in B and T cell epitope recognition would offer the opportunity to use N-terminal fusion insertions to specifically stimulate pure T cell responses, in the absence of any B cell response. Further experiments have identified other permissive sites in the CPV capsid that allow a proper antigen delivery without capsid destruction (Hurtado *et al*., 1996).

The availability of large amounts of these modified VLPs and their high immunogenicity in different animal species offer a promising future as antigen delivery system and for the development of new subunit vaccines useful for several diseases.

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References

Agbandje M, Kajigaya S, McKenna R, Young NS & Rossmann MG (1994) The structure of human parvovirus B19 at 8A resolution. Virology 203: 106–115.

Bansal GP, Hatfield JA, Dunn FE, Kramer AA, Brady F, Riggin CH, Collet MS, Yoshimoto K, Kajigaya S & Young NS (1993) Candidate recombinant vaccine for human B19 parvovirus. J. Inf. Dis. 167: 1034–1044.

Brown CS, van Bussel MJAWM, Wassenaar ALM van Elsacker-Niele AMW, Weiland HT & Salimans MMM (1990) An immunofluorescence asay for the detection of parvovirus B19 IgG and IgM antibodies based on recombinant viral antigen. J. Virol. Methods 29: 53–62.

Brown CS, Van Lent JWM, Vlak JM & Spaan WJM (1991) Assembly of empty capsids by using baculovirus recombinants express-

human parvovirus B19 structural proteins. J. Virol. 65: 2702–2706.

Brown CS, Jensen T, Meloen R, Puijk W, Sugamura K, Sato H & Spaan WJH (1992) Localization of an immunodominant domain on baculovirus-produced parvovirus B19 capsids: correlation to a major surface region on the native virus particle. J. Virol. 66: 6989–6996.

Brown CS (1994) Human parvovirus B19. Expression and applications of its structural proteins. Ph.D. Thesis. Leiden University.

Brown CS, Welling-Wester S, Feijlbrief M, Van Lent JWM & Spaan WJH (1994) Chimeric parvovirus B19 capsids for the presentation of foreign epitopes. Virology. **198:** 477–480.

Casal JI, Cortés E, Lopez de Turiso JA & Vela C (1992) Production of porcine and canine parvovirus-like particles using recombinant baculovirus. In: "Baculovirus and recombinant protein production processes" JM Vlak, EJ Schlaeger & AR Bernard (eds) pp. 76–92. Editiones Roche. Basel. Switzerland.

Christensen JT, Storgaard T, Bloch B, Alexandersen S & Aasted B (1993) Expression of aleutian mink disease parvovirus proteins in a baculovirus vector system. J. Virol. 67: 229–238.

Christensen J, Alexandersen S, Bloch B, Aasted B & Uttenthal A (1994) Production of mink enteritis parvovirus empty capsids by expresion in a baculovirus vector system: a recombinant vaccine for mink enteritis virus. J. Gen. Virol. 75: 149–155.

Cortés E, SanMartin C, Langeveld J, Meloen R, Dalsgaard K, Vela C & Casal JI (1993) Topographical analysis of canine parvovirus virions and recombinant VP2 capsids. J. Gen. Virol. 74: 2005–2010.

Cotmore SF & Tattersall P (1987) The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. 33: 91-174.

Gray JJ, Cohen BJ & Desselberger U (1993) Detection of human parvovirus B19-specific IgM and IgG antibodies using a recombinant viral VP1 antigen expresse in insect cells and stimation of time of infection by testing for antibody avidity. J. Virol. Methods 44: 11–24.

Hurtado A, Rueda P, Nowicky J, Sarraseca J & Casal JI (1996) Identification of domains in canine parvovirus VP2 essential for the assembly of virus-like particles. J. Virol. In press.

Joo HS, Donaldson-Wood CR & Johnson RHA (1976) A standardized hemagglutination-inhibition test for porcine parvovirus. Aust. Vet. J. 52: 422–424.

Kajigaya S, Fuji H, Field A, Anderson S, Rosenfeld S, Anderson LJ, Shimada T & Young NS (1991) Self-assembled B19 parvovirus capsids, produced in a baculovirus system are antigenically and immunogenically similar to native virions. Proc. Natl. Acad. Sci. USA 88: 4646–4650.

Kool M, Voncken JW, Van Lier FLJ, Tramper J & Vlak JM (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology 183:739–746.

Kurtzman GJ, Cohen BJ, Field AM, Oseas R, Blaese M & Young NS (1989) Immune response to B19 parvovirus and an antibody defect in persistent viral infection. J. Clin. Invest. 84:1114–1123.

Langeveld J, Casal JI, Vela C, Dalsgaard K, Smale SH, Puijk WC & Meloen R (1993) B cell epitopes of canine parvovirus: distribution on the primary structure and exposure on the viral surface. J. Virol. 67: 765–772.

Langeveld J, Kamstrup S, Uttenthal A, Strandbygaard B, Vela C, Dalsgaard K, Beekam N, Meloen R & Casal JI (1995) Full protection in mink against mink enteritis virus with new generation canine parvovirus vaccines based on synthetic peptides or recombinant protein. Vaccine. 13: 1033–1037.

Lopez de Turiso JA, Cortés E, Ranz A, Garcia J, Sanz A, Vela C and Casal JI (1991) Fine mapping of canine parvovirus B cell epitopes. J. Gen. Virol. 72: 2445–2456.

Lopez de Turismo JA, Cortés E, Martinez C, Ruiz de Ybañez R, Simarro I, Vela C & Casal JI (1992) Recombinant vaccine for canine parvovirus in dogs. J. Virol. 66: 2748–2753.

Luckow VA & Summers MD (1988) Trends in the development of baculovirus expression vectors. Bio/Technology 6: 47–55.

Luckow V (1991) Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: Recombinant DNA technology and applications, A Procop, RK Bajpai & CS Ho (eds) pp. 97–153. McGraw-Hill, Inc. New York.

Martinez C, Dalsgaard K, Lopez de Turismo JA, Cortés E, Vela C & Casal JI (1992) Construction of porcine parvovirus empty capsids with high immunogenic activity. Vaccine 10: 684–690.

Matsuura Y, Possee RD, Overton HA & Bishop DHL (1987) Baculovirus expression vectors: The requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. 68: 1233-1250.

Miyamura K, Kajigaya S, Momoeda M, Smith-Gill SJ & Young NS (1994) Parvovirus particles as platforms for protein presentation. Proc. Natl. Acad. Sci. USA 91: 8507–8511.

Rosenfeld SJ, Yoshimoto K, Kajigaya S, Anderson S, Young NS, Field A, Warrener P, Bansal G & Collet MS (1992) Unique region of the minor capsid protein of human parvovirus B19 is exposed on the virion surface. J. Clin. Invest. 89: 2023–2029.

Ruffing M, Zentgraf H & Kleinschmidt JA (1992) Assembly of viruslike particles by recombinant structural proteins of adeno-associated virus type 2 in insect cells. J. Virol. 66: 6922–6930.

Saikawa T, Anderson S, Momoeda M, Kajigaya S & Young NS (1993) Neutralizing linear epitopes of B19 parvovirus cluster in the VP1 unique and VP1-VP2 junction regions. J. Virol. 67: 3004–3009.

Saliki JT, Mizak B, Flore HP, Gettig RR, Burand JP, Carmichael LE, Wood HA & Parrish CR (1992) Canine parvovirus empty capsids produced by expression in a baculovirus vector: use in analysis of viral properties and immunization of dogs. J. Gen. Virol. 61: 33–41.

Salimans MMM, van Bussel MJAWM, Brown CS & Spaan WJM (1992) Recombinant parvovirus B19 capsids as a new substrate for detection of B19 specific IgG and IgM antibodies by an enzyme-linked immunosorbent assay. J. Virol. Methods 39: 247–258.

Sato H, Hirata J, Kuroda N, Shiraki Y, Maeda Y & Okochi K (1991) Identification and mapping of neutralizing epitopes of human parvovirus B19 by using human antibodies. J. Virol. 66: 106–114.

Schwartz TF, Roggendorf M & Deinhardt F (1988) Human Parvovirus B19: ELISA and immunoblot assays. J. Virol. Methods 20: 155–168.

Sedlik C, Sarraseca J, Rueda P, Leclerc C & Casal JI (1995) Immunogenicity of poliovirus B and T cell epitopes presented by hybrid porcine parvovirus particles. J. Gen. Virol. 76: 2361–2368.

Sisk WP & Berman ML (1987) Expression of human parvovirus B19 structural protein in *E. coli* and detection of antiviral antibodies in human serum. Bio/Technology 5: 1077–1080.

Tsao J, Chapman MS, Agbandje M, Keller W, Smith K, Wu H, Luo M, Smith TJ, Rossmann MG, Compans RW & Parrish CR (1991) The three dimensional structure of canine parvovirus and its functional implications. Science 251: 1456–1464.

Tullis G, Burger L & Pintel DJ (1991) MVM VP2 is both required and sufficient for the production of infectious virus. Abstracts 4th Parvovirus Workshop. Elsinore. Denmark.

Vialard J, Lalumiere M, Vernet T, Briedis D, Alkhalib G, Henning D, Levin D & Richardson C (1990) Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novle baculovirus vector containing the β-galactosidase gene. J. Virol. 64: 37–50.

Vlak JM, Schouten A, Usmany M, Belsham GJ, Klinge-Roode EC, Maule AJ, Van Lent JWM & Zuidema D. (1990) Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. Virology 179: 312–320.

Zuidema D, Schouten A, Usmany M, Maule AJ, Belsham GJ, Roosien J, Klinge-Roode EC, Van Lent JWM & Vlak J (1990) Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. J. Gen. Virol. 71:2201–2209.

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Classical swine fever virus diagnostics and vaccine production in insect cells

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Key words: classical swine fever virus, envelope glycoproteins, baculovirus expression, subunit vaccines, diagnostic tests

Introduction

Classical swine fever virus (CSFV; synonym hog cholera virus), bovine viral diarrhoea virus (BVDV), and border disease virus (BDV), members of the Pestivirus genus of the family of *Flaviviridae* (Francki *et al.*, 1991) are small, enveloped, positive-stranded RNA viruses (Moennig & Plagemann, 1992). The viruses are structurally, antigenically and genetically closely related. BVDV and BDV can infect ruminants and pigs. CSFV infections are restricted to pigs. The envelope of the CSF virion contains three glycoproteins, El (gp51 to gp54), E2 (gp42 to gp46), and E3 (gp31) (Moormann *et al.*, 1990; Thiel *et al.*, 1991). Animals infected with pestiviruses raise antibodies against at least two viral glycoproteins, namely El and E2 (Kwang *et al.*, 1992; Terpstra & Wensvoort, 1988). El is the most immunodominant protein present in the envelope and plays an important role in virus neutralization (Wensvoort *et al.*, 1990).

CSFV causes a highly contagious and often fatal disease in pigs. Outbreaks of the disease occur intermittently in several European countries and can cause large economical losses. An active serological screening program followed by "stamping out" of infected herds, is used to control outbreaks of the disease. This policy is not only expensive but is also not sufficient for eradication of classical swine fever in Europe. Vaccination of pigs with a live attenuated CSFV vaccine strain, the C strain, protects pigs from classical swine fever (Terpstra & Wensvoort, 1988). However, pigs vaccinated with conventional vaccines like the C strain, can not be discriminated serologically from pigs infected with field strains. These vaccines are therefore not suitable for a controlled eradication of classical swine fever. The use of a subunit vaccine, e.g. on the basis of glycoprotein El, would allow such differentiation. Pigs infected with field virus could be discriminated from pigs vaccinated with E1 by detection of antibodies specifically directed against E2 or other immunogenic (non)-structural viral proteins. Results of vaccination studies in pigs with a pseudorabies virus (PRV) vector expressing the El protein showed that an antibody response directed against E1 is sufficient to protect pigs from classical swine fever (van Zijl *et al.*, 1991). However, the use of live virus recombinant vaccines in the field is still a matter of controversy. Therefore the use of a non-replicating El subunit vaccine in pigs would be advantageous. Induction of a protective immune response in the natural host by a non-replicating vaccine, however, is only successful when large amounts of viral antigen are applied. The baculovirus-insect cell system, which supports high-level production of properly processed heterologous proteins, is therefore the most suitable system to produce a non-replicating El subunit vaccine. In this review we describe the expression of glycoprotein El of CSFV by the baculovirus-insect cell system and the application of this El as a subunit vaccine for CSFV (Hulst *et al.*, 1993). In addition some aspects of the use of El and E2, produced in insect cells, in diagnostic tests are discussed.

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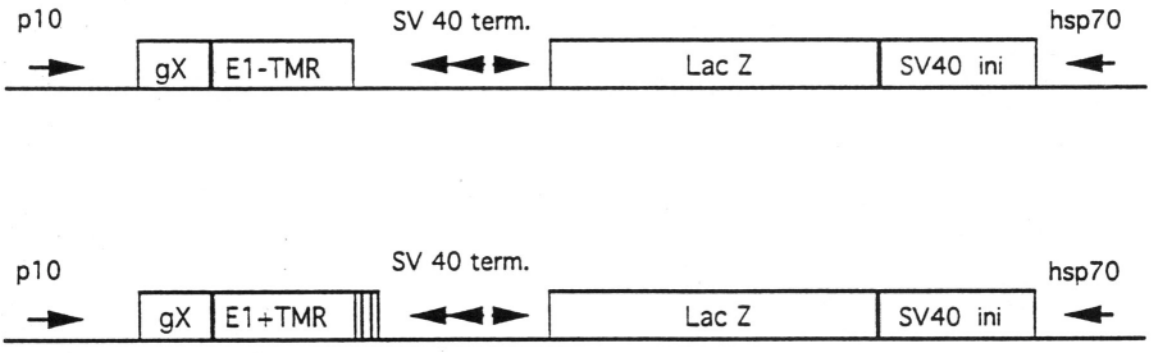

Figure 1. Organization of the p10 locus of the BacE1[-] and BacE1[+] virus (Vlak *et al.*, 1990); p10: p10 promoter; hsp 70: D. melanogaster hsp 70 promoter; SV40 ini: SV40 ATG; LacZ: *E coli* LacZ gene; SV40 term.: SV40 transcription termination sequence; gX: pseudorabies virus gX signal sequence; E1+TMR and E1-TMR: E1 gene of the CSFV strain Brescia with (+TMR) and without (-TMR) a C-terminal transmembrane anchor. Arrows indicate the direction of transcription of the p10, and hsp 70 promoter. Arrowheads indicate the orientation of the transcription termination signals.

Expression and characterization of E1

Our approach to express E1 with the baculovirus system was based on our experiences with expression of this protein with the PRV vector (Van Zijl *et al.*, 1991). Two versions of the E1 gene of classical swine fever strain Brescia (Moormann *et al.*, 1990), one encoding E1 with a C-terminal membrane anchor (transmembrane region, TMR), and one encoding E1 without a C-terminal TMR, were inserted into the PRV glycoprotein gX locus (Rea *et al.*, 1985), and expressed in swine kidney cells. E1 without TMR was secreted, whereas E1 with TMR was retained in the membranes of the endoplasmic reticulum. To express both versions of the Brescia E1 gene in Sf21 insect cells, the sequences encoding E1 with and without TMR were fused to the signal sequence of gX of PRV and inserted into the p10 locus of *Autographa californica* nuclear polyhedrosis virus (AcNPV), using pAcAS3 (Vlak *et al.*, 1990) as transfer vector (Fig. 1). Polyhedrin-positive plaques expressing β-galactosidase were isolated and tested for expression of E1 by immunostaining with an E1 specific monoclonal antibody (Wensvoort *et al.*, 1989). One plaque purified E1-TMR virus (BacE1[-]) and one plaque purified E1+TMR virus (BacE1[+]) were selected for further characterization by radio immunoprecipitation (Fig. 2).

The E1 protein precipitated from the BacE1 [+] cell-lysate was similar in size to wild-type E1 (compare lanes 1 and 6; wild-type E1 is a doublet with a molecular mass of 51 to 54 kD (Wensvoort *et al.*, 1990)). As expected the E1 protein precipitated from the BacE1 [-] lysate (lane 4) was slightly smaller (49 to 52 kD) than wild type E1. E1-TMR was secreted from insect cells (lane 5) with a somewhat lower molecular weight (46 to 48 kD) than cell-associated E1-TMR. No E1+TMR protein was secreted from insect cells (lane 7).

The N-glycans of the cell-associated E1+TMR were completely sensitive to endo H, indicating that similarly as in CSFV infected cells, the E1+TMR protein is anchored in the membranes of the endoplasmic reticulum or cis-Golgi region of insect cells (Hulst *et al.*, 1993). The N-linked glycans of the secreted E1 were partially resistant to endo H, indicating that a part of the high mannose units were trimmed to a smaller endo H resistant form (Kuroda *et al.*, 1990). This explains the lower molecular weight of the secreted E1 -TMR (lane 5) compared to cell associated E1 -TMR (lane 4).

Similarly as in CSFV infected cells (Wensvoort *et al.*, 1990; Thiel *et al.*, 1991), E1 secreted from insect cell was efficiently dimerized. Furthermore, E1+TMR and both the secreted and cell-associated forms of E1 -TMR reacted identically as native E1 in an ELISA with four E1 specific monoclonal antibodies, which each recognize a discontinuous antigenic domain on E1 (van Rijn *et al.*, 1993). This indicated that E1 expressed in insect cells is antigenically indistinguishable from native E1.

Production level of E1

The level of expression of E1 in Sf21 cells and in the medium, determined at different time points after infection in an E1 specific ELISA (Wensvoort *et al.*,

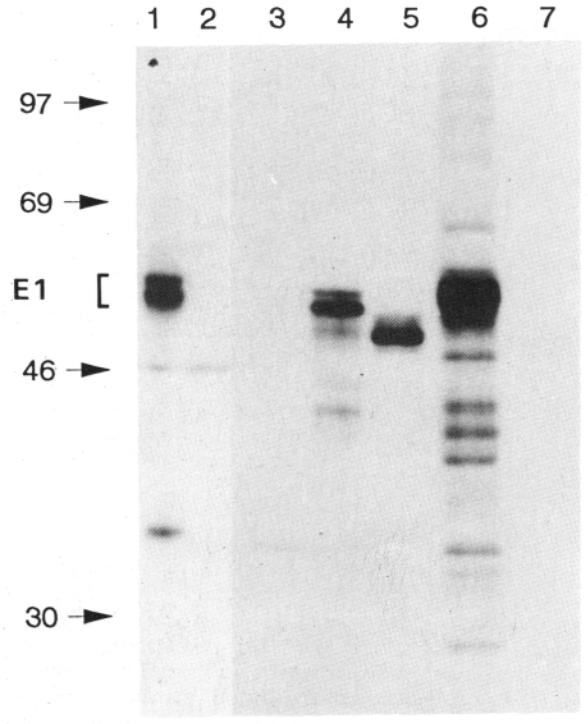

Figure 2. Radio immunoprecipitation assay with a mixture of three E1 specific monoclonal antibodies (Mabs 3, 6 and 8; Wensvoort *et al.*, 1989) of media and lysates of Sf21 cells infected with BacE1[-], BacE1[+] and wild-type AcNPV. Cells were labeled at 44 h after infection with 40 μCi/ml of [^{35}S]methionine for 6 h. Immune-precipitates were analyzed in a 10% SDS-PAGE and visualized by autoradiography. Wild type E1, immuno-precipitated from the lysate of [^{35}S]cysteine labeled SK6 cells infected with CSFV strain Brescia (van Zijl *et al.*, 1991) was run in parallel; lane 1, AcNPV cell lysate; lane 2, AcNPV medium; lane 3, BacE1[-] cell lysate; lane 4, BacE1[-] medium; lane 5, BacE1[+] cell lysate; lane 6, BacE1[+] medium; lane 7. Molecular weight calibration (10^3) is indicated to the left of the autoradiograph.

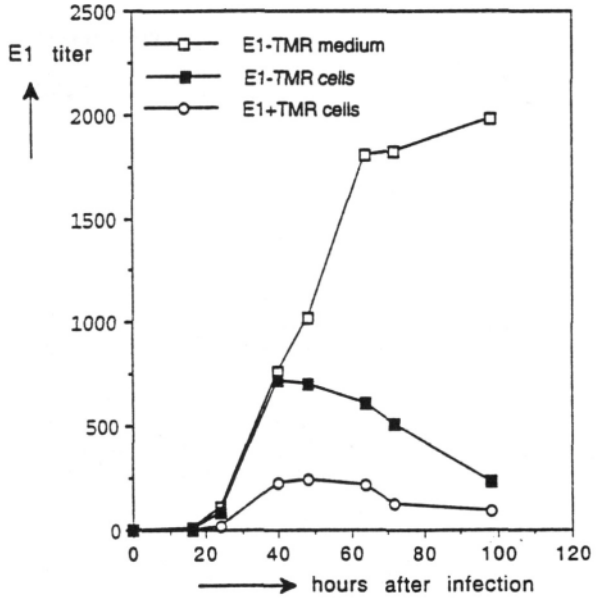

Figure 3. Time course of E1 production in Sf21 cells infected with BacE[-] and BacE1[+]. ELISA titers of E1 in the medium (E1-TMR) and in 1% Nonidet-P40 treated cells (E1-TMR and E1+TMR) at 16, 24, 40, 48, 64, 72 and 98 h after infection.

1988), showed that the total amount of E1-TMR (cell-associated plus secreted forms) synthesized in Sf21 cells is about 10 times higher than for E1+TMR (Fig. 3.) Because no significant growth differences between BacE1[-] and BacE1[+] virus were observed and the level of E1 specific RNA present in Sf21 cells infected with both viruses was almost identical (Fig. 4), this lower production level of E1+TMR is probably due to inhibition of protein synthesis as a result of the accumulation of E1+TMR in the membranes of the endoplasmic reticulum (see above).

On the basis of this ELISA, it was estimated that 75% of the E1-TMR protein was secreted from insect cells. The concentration of E1 secreted in the medium, determined by ELISA using a pure E1 preparation as standard, was approximately 30 to 50 μg/10^6 cells (50 to 90 μg/ml) when Sf21 cells were grown as monolayers in serum-free medium (SF900; Gibco BRL).

In a pilot experiment, *Spodoptera exiqua* larvae were infected with BacE1[-] and BacE1[+] virus. As expected, only the E1 -TMR protein was secreted in the hemolymph fluid of the larvae. The production level of E1-TMR in the hemolymph fluid was approximately 300 to 400 μg/ml. Analysis of the E1 -TMR protein by SDS-PAGE and Western blotting showed that 50% of the E1 was C-terminally cleaved probably by a host protease to a smaller protein with a molecular weight of 29 to 31 kD (Fig. 5). Because the neutralizing epitopes on E1 are positioned in the N-terminal half of the protein (Van Rijn *et al.*, 1993), E1 produced in larvae could still be suitable as a subunit vaccine. To improve production of E1, further experiments with larvae producing more hemolymph fluid, are needed. Furthermore, vaccination experiments in pigs with E1 secreted in the hemolymph fluid should be performed to establish whether E1 produced in larvae is as efficacious in inducing protection against CSFV as E1 produced in insect cells.

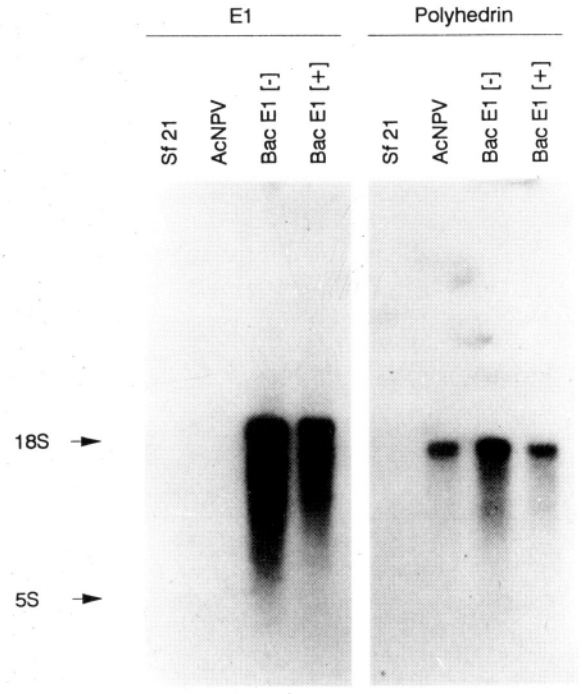

Figure 4. Northern blot carrying equal amounts (15 μg) of cytoplasmic RNA isolated at 40 h after infection from cells infected with AcNPV, BacE1[-] and BacE1[+]. RNA from mock-infected Sf21 cells was run in parallel as a control. The blot was hybridized with a ^{32}P labeled nick-translated E1 specific probe, exposed and washed. The blot was re-probed with a ^{32}P labeled nick-translated polyhedrin specific probe to account for gene-doses effect. Markers; 18S and 5S ribosomal RNA are indicated by arrowheads.

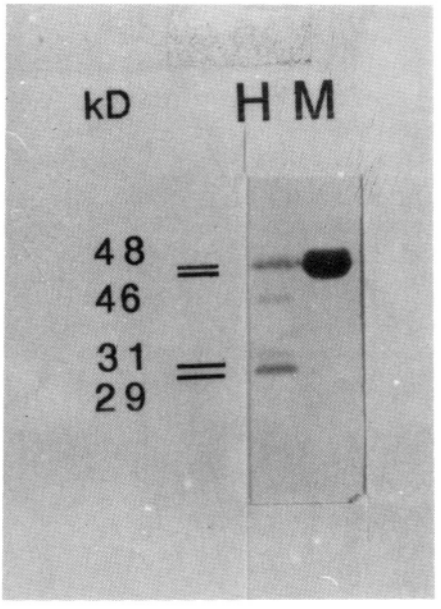

Figure 5. Western blot analyses of E1 secreted in the hemolymph fluid (H) of larvae infected with BacE1[-] and secreted in the medium (M) of Sf21 cells infected with BacE1[-]. E1 was detected using a monoclonal antibody specifically directed against E1 of CSFV strain Brescia (Wensvoort *et al.*, 1990). The molecular weights of the E1 proteins are indicated to the left of the blot.

Vaccination of pigs with E1 produced in insect cells

For the first vaccination trial with E1 produced with the baculovirus system, E1-TMR purified from the medium of insect cells by immunoaffinity chromatography, was used (Hulst *et al.*, 1993). Because no data were available regarding the dose of E1 required for the induction of a protective immune response in pigs by a dead subunit vaccine, groups of pigs were vaccinated with high doses, i.e., 20 and 100 μg of E1. The vaccine was applied in a double water-oil emulsion (Herbert, 1965; Barterling & Vreeswijk, 1991). After vaccination all pigs developed high neutralizing antibody liters against classical swine fever virus (Table 1). In fact, no significant differences in liters were observed between pigs vaccinated with 20 or 100 μg of E1 42 days after inoculation. After intranasal challenge with 100 LD_{50} (50% lethal dose) of classical swine fever virus strain Brescia, all pigs vaccinated with E1 were completely protected and did not show any signs of disease or viraemia. In contrast, the non-vaccinated pigs developed fever from day 4 and 5 on, became recumbent at day 6, and were killed when moribund at day 8 post challenge.

The results of this limited vaccination experiment with E1 are highly promising in several aspects. Firstly, after vaccination with the conventional Chinese (C)-strain vaccine, pigs with neutralizing antibody titers of 30 or higher are protected from classical swine fever, and do not transmit the virus to seronegative contact pigs, after challenge with virulent CSFV (Terpstra *et al.*, 1988). Pigs inoculated once with 20 μg of E1 produced in insect cells, develop neutralizing antibody titers of 3000 and higher, indicating that a much lower dose of E1 might be sufficient to induce protection against CSF. Preliminary results with lower doses of E1, directly prepared from serum-free SF900 insect cell medium, confirm that the dose needed to protect pigs, and to prevent transmission of classical swine fever virus, is significantly lower than 20 μg E1 (the 50% protective dose is about 2 μg E1).

Secondly, the rise of antibody titers against E1 proceeded much faster in pigs vaccinated with E1 produced in insect cells (dead E1) than in pigs infected

Table 1. Vaccination of pigs with E1[1]

Dose	Pig	Neutralizing antibody titer[2]				Challenge[3]		
(μgr)		day0	day14	day28	day42	disease	viremia	death
20	1	<6.25	150	4800	>6400	–	–	–
20	2	<6.25	25	3200	4800	–	–	–
100	3	<6.25	25	2400	2400	–	–	–
100	4	<6.25	19	2400	4800	–	–	–
none	5	<6.25	<6.25	<6.25	<6.25	+	+	+
none	6	<6.25	<6.25	<6.25	<6.25	+	+	+

[1] Specific pathogen free pigs were vaccinated on day 0 and challenged with CSFV strain Brescia on day 42. After challenge, pigs were observed daily for signs of disease, and body temperatures were measured.
[2] The neutralizing antibody titer is expressed as the reciprocal of the serum dilution effectively neutralizing 100 50% tissue culture infective dose of CSFV strain Brescia.
[3] Fever, anorexia, thrombocytopenia, and paralysis are regarded as signs of disease. Viraemia was established by determining classical swine fever virus titers in the leucocyte fraction of the blood on days 38, 40, 42, 45, 47, 49, 52, 54, and 56.

with low-virulent field strains of classical swine fever virus or with the PRV-E1 recombinant viruses (Van Zijl *et al.*, 1991; G. Wensvoort, personal communication). All these observations indicate that E1 expressed by the baculovirus-insect cell system may be a very effective dead subunit vaccine.

Thirdly, pigs infected with field virus can be discriminated from pigs vaccinated with E1 on the basis of presence (field virus infection) or absence (vaccination with E1) of antibodies directed against other immunogenic structural or non-structural viral proteins. Such a discriminating serological test has to be developed and should accompany the E1 subunit vaccine in a vaccination campaign for a controlled eradication of classical swine fever during outbreaks of the disease.

Application of CSFV proteins expressed with a baculovirus vector in diagnostic tests

The pestiviruses CSFV, BVDV and BDV are structurally, antigenically, and genetically, closely related. The genomic organization of these viruses are similar. Sera induced in pigs infected with CSFV and in pigs and ruminants infected with BVDV, cross-react in neutralization assays (Wensvoort *et al.*, 1989). Therefore, a serological test which specifically detects antibodies in field sera directed against CSFV specific epitopes, is essential to differentiate between pigs infected with CSFV and pigs infected with BVDV or BDV. For CSFV, such a serological test (Wensvoort *et al.*, 1988) is available commercially for several years already. In this diagnostic ELISA, which detects antibodies in field sera, which are specifically directed against conserved (present on all CSFV strains) epitopes on E1 of CSFV (Wensvoort, 1989), native E1 was used as antigen. This antigen, however, has now been replaced by E1 synthesized in insect cells. Replacement of native El appeared to have several advantages. Instead of intensive large scale isolation of native E1 from porcine kidney cells infected with CSFV, serum-free medium of Sf21 cells infected with BacE1[-], grown in small culture flasks (75 cm^2), can be used directly in this assay. Because of the constant quality of the E1 produced in insect cells, 75% less inconclusive results with field sera were scored in this ELISA. Furthermore, E1 synthesized in insect cells improved the sensitivity of the test (R. Bloemraad, personal communication).

Animals infected with pestiviruses also raise antibodies against envelope glycoprotein E2 (Kwang *et al.*, 1992). Glycoprotein E2 of CSFV, recently identified as a ribonuclease, can also be expressed in large amounts in insect cells (Hulst *et al.*, 1994). The ribonuclease specific activity of E2 produced in insect cells is comparable to that of native E2 (Schneider *et al.*, 1993), indicating that the conformation of E2 synthesized in insect cells is indistinguishable from native E2. Therefore, E2 synthesized in insect cells could also be suitable as antigen in an serological test for the detection of field infections. Preliminary results in our laboratory show that such a test can indeed be developed on the basis of E2. Nevertheless, before a reliable, sensitive, and CSFV specific serological test based on E2 produced in insect cells can be employed commercial-

ly, monoclonal antibodies, specifically directed against conserved epitopes (present on all CSFV strains) on E2 of CSFV, have to be produced. For the production of these monoclonal antibodies, properly synthesized, and processed viral proteins synthesized in insect cells should be very suitable.

Because animals infected with pestiviruses also raise antibodies to the non-structural viral protein, p80 (Donis & Dubovi, 1987), a diagnostic test for the detection of field infections could also be developed on the basis of this protein. Using p80 expressed in insect cells as antigen in an ELISA, Petric *et al.* (1992) and Vanderheijden *et al.* (1993), showed that anti-BVDV antibodies could be detected in field sera from cattle infected with BVDV. Because the degree of homology between the amino acid sequences encoding p80 of BVDV, BDV and CSFV is high (more than 86%; Moormann *et al.*, 1990), sera from pigs infected with BVDV and BDV cross-react with CSFV p80. Therefore, a serological test on the basis of p80 will not be suitable to differentiate between pigs infected with CSFV, BVDV and BDV.

Concluding remarks

The baculovirus-insect cell expression system has provided us with properly synthesized envelope proteins E1 and E2 of CSFV. Because the production level in insect cells of these proteins is high, we are able to apply these expression products for various purposes. Nevertheless, in the literature their are many examples that the production levels of viral proteins in the baculovirus insect cell system can be poor. In our laboratory, the production levels of the structural proteins of porcine reproductive and respiratory syndrome virus (Lelystad virus) in insect cells were poor compared to CSFV E1 and E2 (J. Meulenberg, personal communication). Instability of the messenger RNAs of the viral proteins in the nuclei of insect-cells or instability of the proteins themselves, as a result of a different processing or routing in insect cells compared to mammalian cells, may be responsible for this low level of production. It therefore should be recognized that RNA viruses like CSFV and Lelystad virus replicate in the cytoplasm of the mammalian cell. For the production of proteins of these kind of viruses, eukaryotic expression systems based on vectors replicating in the cytoplasm, like the semliki forest virus vector system (Liljeström & Garroff, 1991), or the vaccinia virus vector system (Mackett *et al.*, 1982), might be more suitable.

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We thank Just Vlak and John Martens for providing the *Spodoptera exiqua larvae* and for their help to infect the larvae with the baculovirus-E1 recombinants. The data of the diagnostic ELISA using E1 were kindly provided by Rinus Bloemraad.

Addendum in proof

In this paper we still used the old pestivirus protein nomenclature. A new nomenclature of these proteins will, however, be proposed to the International Committee on Taxonomy of Viruses by the Flaviviridae Study Group. In recent papers this new nomenclature has already been used by several research groups. The nomenclature of pestivirus proteins used in this paper compared to the new one is as follows: E1 is renamed E2, E2 is renamed E^{rns}, E3 is renamed E1, and p80 is renamed NS3.

References

Barteling S & Vreeswijk J (1991) Developments in foot and mouth disease vaccines. Vaccine 9: 75–88.

Donis R & Dubovi E (1987) Molecular specificity of the antibody responses in cattle naturally and experimentally infected with cytopathic and noncytopathic bovine viral diarrhea virus biotypes. American Journal of Veterinary Research 48: 1549–1554

Francki R, Fauquet C, Knudson D & Brown F (1991) Flaviviridae Arch. Virol. Suppl. 2: 223–233.

Herbert W (1965) Multiple emulsions: A new form of mineral-oil antigen adjuvant. Lancet 11: 771

Hulst M, Westra D, Wensvoort G & Moormann R (1993) Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. J. Virol. 67: 5435–5442.

Hulst M, Himes G, Newbigin E & Moormann R (1994) Glycoprotein E2 of classical swine fever virus: expression in insect cells and identification as a ribonuclease. Virology 200: 558–565.

Kuroda K, Geyer H, Geyer R, Doerfler W & Klenk H (1990) The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. Virology 174: 418–429.

Kwang J, Littledike E, Donis R & Dubovi E (1992) Recombinant polypeptide from the gp48 region of the bovine viral diarrhoea virus (BVDV) detects serum antibodies in vaccinated and infected cattle. Vet. Microbiol. 32: 281–292.

Liljeström P & Garoff H (1991) A new generation of animal cell expression vectors based on the Semliki forest virus replicon. Biotechnology 9: 1356–1362.

Mackett M, Smith G & Moss B (1982) Vaccinia virus expression vector. PNAS 79: 7415–7419.

Moennig V & Plagemann (1992) The pestiviruses. Adv. Virus Res. 41:53–98

Moormann R, Warmerdam P, van der Meer B, Schaaper W, Wensvoort G & Hulst M (1990) Molecular cloning and nucleotide

sequence of hog cholera virus strain Brescia and mapping of the genomic region encoding envelope protein E1. Virology 177: 184–198.

Petric M, Yolken R, Dubovi E, Wiskerchen M & Collett M (1992) Baculovirus expression of pestivirus non-structural proteins. J. Gen. Virol. 73: 1867–1871.

Rea T, Timmins G, Long G & Post L (1985) Mapping and sequence of the gene for the pseudorabies virus glycoprotein which accumulates in the medium of infected cells. J. Virol. 54: 21–29.

Schneider R, Unger G, Stark R, Schneider-Scherzer E & Thiel H-J (1993) Identification of a structural glycoprotein of a RNA virus as a ribonuclease. Science 261: 1169–1171.

Terpstra C & Wensvoort G (1988) The protective value of vaccine induced neutralizing antibody titres in swine fever. Vet. Microbiol. 16: 123–128.

Thiel H-J, Stark R, Weiland E, Rümenapf T & Meyers G (1991) Hog cholera virus: Molecular composition of virions from a pestivirus. J. Virol. 65: 4705–4712.

Vanderheijden N, De Moerloze L, Vandenbergh D, Chappuis G, Renard A & Lecomte C (1993) Expression of the bovine viral diarrhoea virus Osloss p80 protein: its use as antigen for cattle serum antibody detection. J. Gen. Virol. 74: 1427–1431.

Van Rijn P, van Gennip R, de Meyer E & Moormann R (1993) Epitope mapping of envelope glycoprotein El of hog cholera virus strain Brescia J. Gen. Virol. 74: 2053–2060

Van Zijl M, Wensvoort G, de Kluyver E, Hulst M, van der Gulden H, Gielkens A, Berns A & Moormann R (1991) Live attenuated pseudorabies virus expressing envelope glycoprotein El of hog cholera virus protects swine against both Pseudorabies and Hog Cholera. J. Virol. 65: 2761–2765.

Vlak J, Schouten A, Usmany M, Belsham G, Klinge-Roode M, Maule A, van Lent J & Zuidema D (1990) Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. Virology 179: 312–320.

Wensvoort G, Terpstra C & de Kluyver E (1989) Characterization of porcine and some ruminant pestiviruses by cross-neutralization. Vet. Microbiol. 20: 291–306.

Wensvoort G, Bloemraad R & Terpstra C (1988) An enzyme immuno assay employing monoclonal antibodies and detecting specifically antibodies to classical swine fever virus. Vet. Microbiol. 17: 129–140.

Wensvoort G (1989) Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. J. Gen. Virol. 70: 2865–2876.

Wensvoort G, Boonstra J & Bodzinga B (1990) Immuno-affinity purification of envelope protein El of hog cholera virus. J. Gen. Virol. 71: 531–540.

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Production of multidomain complement glycoproteins in insect cells

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Key words: baculovirus, complement activation, genetic engineering, mosaic protein, serine-protease, zymogen

Abbreviations: AcMNPV – Autographa californica nuclear polyhedrosis virus; C1 – first component of complement; Clq, Clr, Cls – subcomponents of Cl; CCP – complement control protein; EGF – epidermal growth factor; High5 – Trichoplusia ni cell line; SCR – short concensus repeat; Sf9 – Spodoptera frugiperda 9 cell line.

Introduction

The scarcity of sources makes the isolation of most human proteins, peptides and proteinaceous materials impossible or impractical. On the other hand there exists a great demand for complex mammalian glycoproteins for research, diagnosis or therapy. Glycoproteins of various complexity have been expressed in various laboratories (see proceddings of the Workshop on baculovirus and recombinant protein production processes, edited by Vlak *et al.*, 1992) using the baculovirus expression system. Since insect cells do almost all of the posttranslational modifications associated with mammalian cells, the expressed proteins are expected to be near-authentic concerning their biological activity and antigenicity. To produce complex, glycosylated and processed proteins we have the option of choosing from a number of different expression systems, each of which has a particular inherent set of advantages and disadvantages. Bacterial systems provide important advantages, like the ease of use, low cost and high level of expression, but impose a number of limitations for synthesis of eukaryotic proteins (folding, proteolytic processing, glycosylation, secretion, subunit assembly, etc.). For this reason eukaryotic cells are preferred for expression of mammalian genes. There are several host-vector systems for heterologous gene expression in yeast or mammalian cells. The disadvantages of the mammalian expression systems are the high cost and complexity of the cell culture maintenance and the difficulty of manipulating large viral genomes, as well as the relatively low level of expression, that might be a high price for the correct folding and full biological activity. Insect cell based expression systems are advantageous, since they are cost effective, simple in handling while performing glycosylation, fatty acid acylation, phosphorylation, disulfide formation, C-terminal amidation, β-hydroxylation of aspartate residues, signal peptide cleavage, folding and secretion. Therefore insect cells seem to be suitable for efficient expression of recombinant complex mammalian glycoproteins in biologically active form.

Because of its complexity human complement protein, Cl is an ideal object to test the potential of baculovirus-insect cell expression for producing sophisticated mammalian proteins.

Complement is a complex multicomponent system having many similarities with blood coagulation and fibrinolysis. The classical pathway of complement activation is initiated by the reaction of the first component, Cl, with immune complexes. This recognition leads to removal of the invading bacteria, virus or toxin.

The first component of complement is a supramolecular complex composed of five subcomponents: one Clq, two Clr and two Cls. A specific inhibitor protein, Cl-inhibitor, regulates the activation and function of the C1 complex (reviewed by Arlaud *et al.*, 1987; Schumaker *et al.*, 1987). Cl is a complex multifunctional molecule, and the complexity of its function and regulation is also reflected in the structure of its subunits: Clq is the structural framework of the Cl complex (Reid & Porter, 1976; Borsos *et al.*, 1980) and its interesting flower boquet like structure is shown in Figure 1 and Figure 2. The Ca^{2+}dependent C1sC1rC1rC1s

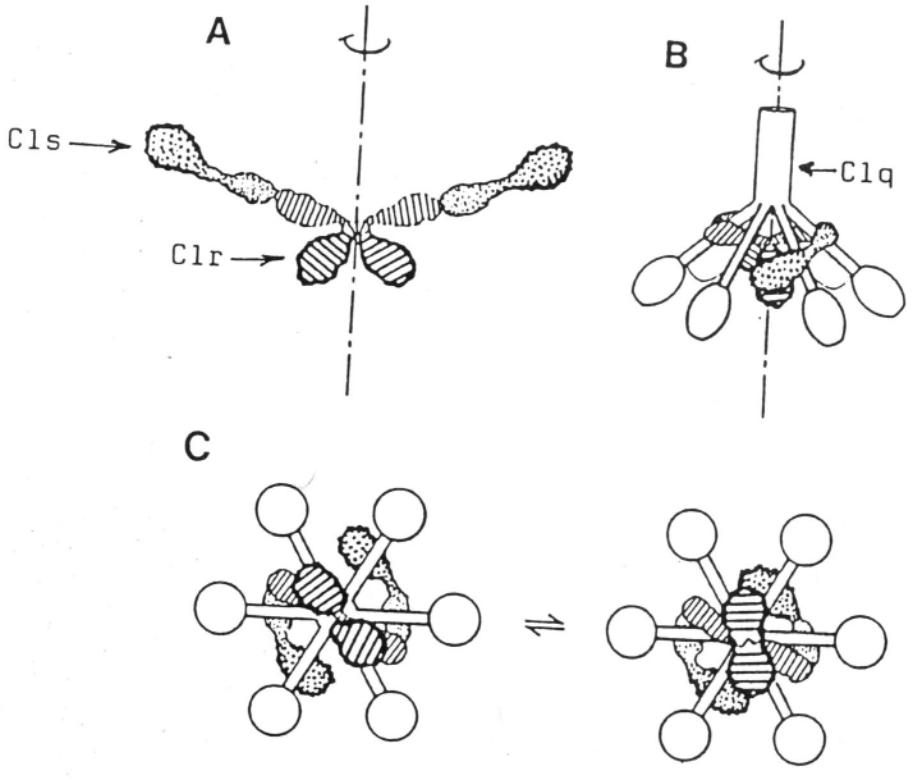

Figure 1. Eclectic functional model of C1. (Adopted from Schumaker *et al*., 1987). (A) The structure of $C1r_2C1s_2$ tetramer. The $C1r_2$ dimer is in the center (crosshatched) and dumbbell-shaped C1s subcomponents (stippled) are located terminally. (B) A side view of the C1, showing the tetramer folded around the stems of C1q. (C) The suggested activation mechanism. On the left: open conformation in which catalytic domains of C1r and C1s are separated. On the right: by C1q arm movement the catalytic domains of C1s are brought into contact with the catalytic domains of C1r.

tetramer is responsible for the enzymatic activity of C1 (Tschopp *et al*., 1980). C1r and C1s are zymogen serine-proteases in the inactive C1 and the tetramer is folded around the collagenous stems of C1q (Figure 1). When C1 binds – through the C1q heads – to immune complexes, the cone formed by the spreading arms will be distorted. This distortion might be the activitation signal that induces a conformational change converting the C1r proenzyme into an enzyme. The activated C1r in turn activates C1s by the cleavage of an Arg-Ile bond. The following initiation of the complement cascade is sustained through the proteolytic action of C1s on C4 and C2.

The C1r and C1s zymogens are single chain glycoproteins (86–78 kD) containing 688 (C1r) and 673 (C1s) amino acid residues (Leytus *et al*., 1986; Tosi *et al*., 1987). There is strong relationship between the two molecules: sequence comparison revealed 40% amino acid identity and conservation of all the cysteine residues (Tosi *et al*., 1987). Both C1r and C1s have been cloned and sequenced and have mosaic like structures typical of plasma serine-proteases (Leytus *et al*., 1986; Tosi *et al*., 1987; Mackinnon *et al*., 1987; Patthy *et al*., 1994). Both C1r and C1s can be divided into six structural motifs (domains or modules), including two pairs of internal repeats (I/III and IV/V), a single copy of motif II and the trypsin like serine-protease module (Figure 3).

Modules I and III are homologous domains, they may be involved in a function that is highly specific to C1r and C1s (for example: Clr-Cls interaction, tetramer-C1q binding). Module II shows homology with epidermal growth factor (EGF). In general such domains are exposed to the extracellular environment and participate in protein-protein interactions. This domain harbours an unusual amino acid, β-hydroxy asparagine in position 150 in C1r and 134 in C1s. The level of hydroxylation in native human C1r is 100%

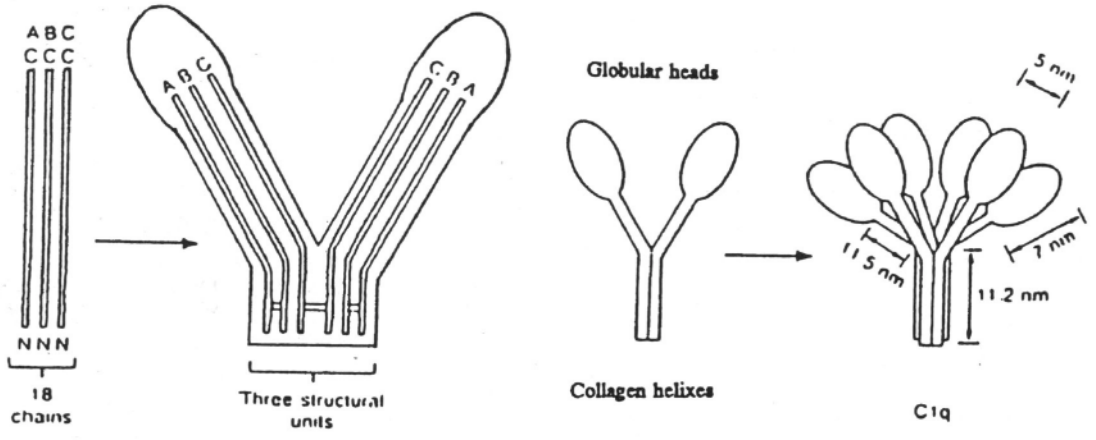

Figure 2. Model of the C1q molecule. The six-stranded C1q subcomponent is the structural framework of the C1 complex. C1q is a large glycoprotein (460 kD) composed of 18 polypeptide chains (6A, 6B, 6C) each of which is approximately 225 amino acid long (Sellar *et al.*, 1991). In each chain a short amino terminal region of 2–11 amino acid is followed by a collagenous segment of 78–84 amino acids, and a carboxy terminal noncollagenous region of 126–133 amino acids. Electronmicroscopic studies have shown C1q to be composed of six globular heads linked via a six collagen-like stalks to a fibril-like central region (Knobel *et al.*, 1975). One copy of each A-, B- and C-chains form a triplet, such that the three collagenous regions form a collagen triple helix, while the C-terminal regions of the three chains form a globular head. Each pair of A- and B-chains within a single triple helix is coupled by a disulfide bond and a second disulfide bridge connects C-chains in adjacent triple helixes to form a pair of helixes and heads. Three such pairs associate noncovalently to form the complete molecule the C1q bouquet (Reid & Porter, 1976).

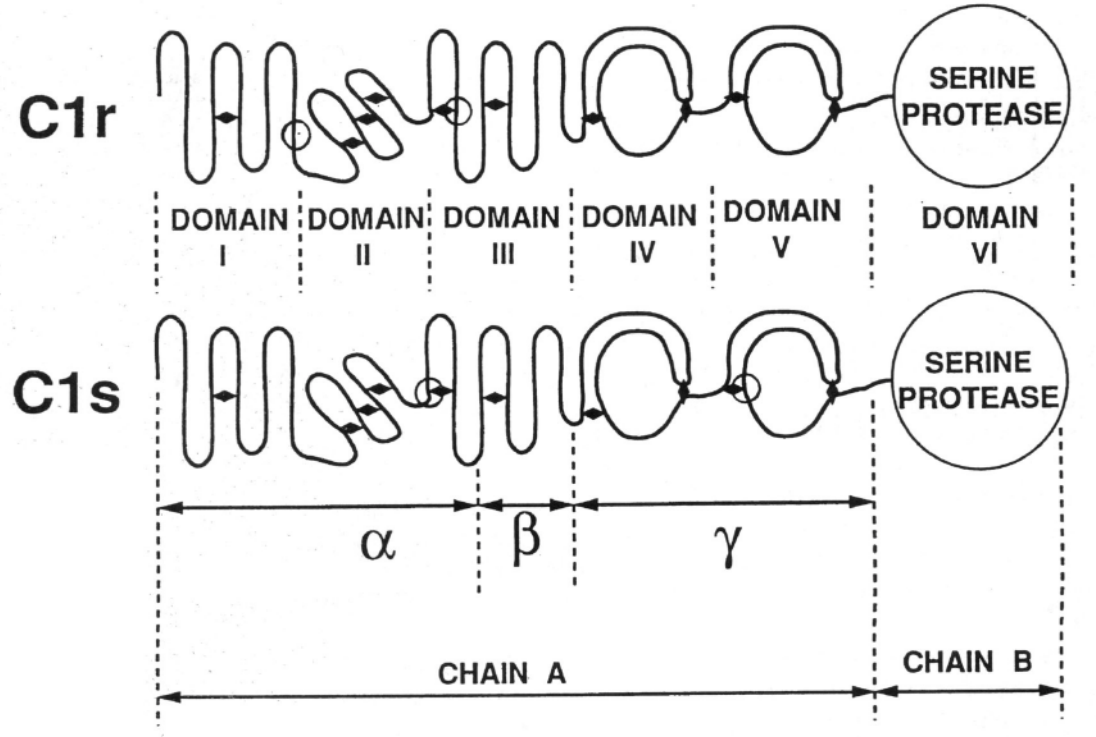

Figure 3. Domain structure for C1r and C1s. Disulphide bridges are indicated by diamonds. There are two glycosylation sites on the non-catalytic regions of both C1r and C1s. (They are showed by circles.) The catalytic domain of C1r contains two additional glycosylation sites. (Partly adopted from Leytus *et al.*, 1986).

while in C1s is only 50% (Thielens *et al*., 1990). The role of this module may be in the Ca^{2+} binding and the tetramer formation. Modules IV–V are homologous motifs (short consensus repeat, SCR or complement control protein, CCP module) and closely associated with the catalytic domain (VI). They are involved in the C1r dimerization and probably in the interaction of C1s with C4 and C2. The serine-protease domains are homologous to the trypsin family. The high specificity of C1r and C1s proteases can be explained by the mosaic structure of these proteins: the five noncatalytic domains determine the unique substrate specificity of the protease domain in these molecules.

The specific inhibitor of C1, a 104 kD plasma glycoprotein is also part of this system. The single chain polypeptide is 478 amino acid residues long (Bock *et al*., 1986) and 49% of the total molecular mass is added as a result of posttranslational glycosylation. C1-inhibitor belongs to the superfamily of serine-protease inhibitors (serpins) and inhibits activated C1r and C1s, as well as prevents spontaneous activation of unactivated C1 (Ziccardi, 1982; Schumaker *et al*., 1989; Tseng *et al*., 1991). It is also critical regulatory component of the coagulation, fibrinolytic and kinin releasing systems. Genetic deficiency of C1-inhibitor, resulting from either quantitative or structural alterations, is the cause for the disease hereditary angiodema (Donaldson & Evans, 1963).

The cDNA and genomic clones are now available for all the C1 subcomponents and C1-inhibitor. We have constructed recombinant Autographa californica nuclear polyhedrosis viruses and expressed the C1r and C1s subunits and C1-inhibitor in Spodoptera frugiperda cells and living lepidopteran larvae, in biologically active form. Individual chains of C1q were also expressed. A systematically designed family of deletion, point and chimeric mutants of the C1r and C1s subunits were also constructed and expressed with the aim to assign binding and regulatory functions to individual modules, and refine the structural-functional model of C1.

The successful expression of such complex multidomain, multifunctional glycoproteins in the baculovirus-insect cell system demonstrates the potential of this system in protein engineering studies with complex mammalian proteins, as well as its capacity as a biotechnological production method.

Expression of C1r and C1s in insect cells

The recombinant virus vector for C1r expression contains the entire coding region of 2115 nucleotide pairs and in addition a noncoding region of 63 base pairs at the 5' end, and a noncoding region of 8 base pairs at the 3' end. This cDNA was placed from HC1r2200 plasmid (Leytus *et al*., 1986) into the transfer vector pAc373 downstream of the strong baculovirus polyhedrin promoter (Gál *et al*., 1989). We have to note here, that in the case of C1r, the elimination of the 5' noncoding region, did not affect the yield of expression.

We used Spodoptera frugiperda (Sf9) cells and Autographa californica nuclear polyhedrosis virus (AcMNPV) as expression system. Recombinant viruses were generated by cotransfection using the calcium-phosphate precipitation method (Summers & Smith, 1987). AcMNPV-C1r viruses were isolated by plaque morphology. The recombinant clones were checked by DNA dot blot and ELISA. C1r produced by the insect cells were secreted into the cell culture medium. Since the protein was recognised by anti-C1r antibodies therefore we could check its level by ELISA and determine its molecular mass by Western blot (Figure 4) (Gál *et al*., 1989).

Using this recombinant virus we could produce 2 μg C1r/10^6 cells in monolayer culture. To produce recombinant protein, cells were routinely grown in 175 qcm flasks in supplemented Grace's medium and the multiplicity of infection was 2. After the infection the medium was changed into serum free Grace's insect medium to avoid the contaminating effect of fetal calf serum on our experiments with C1 subcomponents (also serum proteins). After 3 days post infection the cell culture supernatant was harvested and tested. We followed the expression techniques described by Summers & Smith (1987).

The recombinant virus for C1s contains the 2067-bp coding region and the 293-bp 3'-untranslated region. To avoid possible interference on the expression, the 5'-untranslated sequence was excised in this case (Luo *et al*., 1992). The cDNA was placed into the transfer vector pAcC6 (Luo *et al*., 1992) from pBs-Cls plasmid (Tosi *et al*., 1987). Both the methods used and the level of expression were similar to those described for C1r.

The ratio of the recombinant C1r or C1s to the total protein level in the cell the culture supernatant is about 0.1%. The recombinant Cls was isolated from the cell culture medium by ion exchange chromatography on

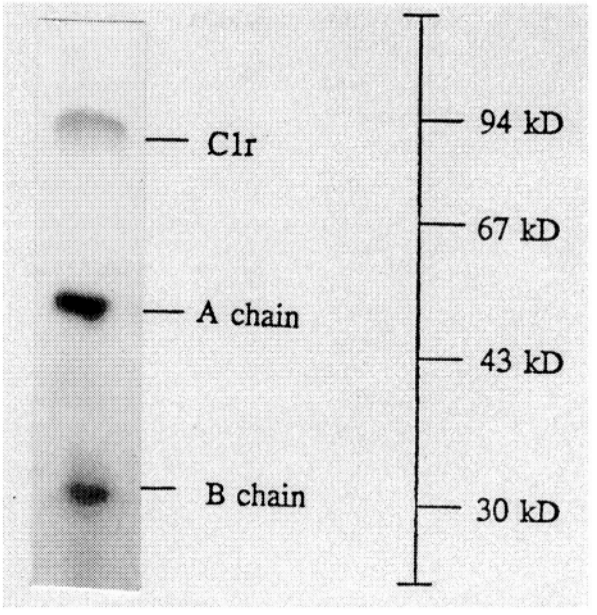

Figure 4. Western blot of the recombinant C1r. (Reducing conditions.) The presence of A- and B-chain is the consequence of the autoactivation of recombinant C1r under our cell culturing conditions. This activation occurs through the clevage of an Arg-Ile bond between the catalytic domain and the N-terminal region of C1r resulting in two, disulfide bridge connected chains of the molecule

DEAE-Sephacel (Luo *et al.*, 1992) taking advantage of the relatively low cost of this method. After a failure with ion exchange chromatography, C1r was purified by affinity chromatography (Monkovic *et al.*, 1992) using the high specifity and the simplicity of this technique.

Biological activity and posttranslational modifications of the recombinant C1r and C1s

The biological activity of the recombinant components was tested by using the hemolytic assay (Rapp & Borsos, 1970). Cl was reconstituted from serum Clq and recombinant tetramer. In this way hemolytically active Cl was generated (Table 1). We believe this was the first report of a sophisticated human zymogen complex produced in functional form by the baculovirus-insect cell expression system (Gál *et al.*, 1989).

Signal peptide cleavage

Since both, recombinant C1r and recombinant C1s were secreted into the medium it is obvious that insect cells can recognize the leader sequences coded by the human cDNA, and process these proteins. The processing includes the cleavage of N-terminal signal peptides (15 amino acids). The sequence analysis of the recombinant C1s showed that its N-terminal is identical to that of the native human serum C1s (Luo *et al.*, 1992).

Glycosylation

On the SDS-PAGE the native and recombinant form of C1r and C1s migrated with a small but significant difference in mobility indicating that the carbohydrate content of the proteins may be different. This difference was significant in the case of the A chain of recombinant C1s while the B chain had the same mobility as the native Cls B chain (Luo *et al.*, 1992). The two glycosylation sites of Cls are both located on the A chain. It has been shown in many cases that insect cells can recognize the N-glycosylation sites of higher eucaryotic proteins, but insect cells usually do not perform complex type glycosylation (Kuroda *et al.*, 1990). In the case of the recombinant proteins the incompletely processed N-linked carbohydrate chains lack at least, the terminal sialic acid residues. This difference however does not reflect in the biological activity of the recombinant C1r and C1s.

β-hydroxylation of Asn in the EGF domain

The posttranslational β-hydroxylation of specific aspartyl and asparaginyl residues has been shown

Table 1. Biological activity of the recombinant C1r and C1s. The C1 molecules, reconstituted from serum C1q with recombinant C1r and C1s, proved to be hemolytically active. Supernatants of mock and wild virus infected cells were used as control

Sample	%-os hemolysis	Hemolytic sites per cell	Concentration of actice C1 molecules
C1q + mock infected cell supernatant	0,10 ± 0,05	0,001 ± $5 \cdot 10^{-4}$	$2{,}8 \cdot 10^{8} \pm 1{,}6 \cdot 10^{8}$ μM
C1q + wild type virus infected cell supernatant	0,10 ± 0,05	0,001 ± $5 \cdot 10^{-4}$	$2{,}8 \cdot 10^{8} \pm 1{,}6 \cdot 10^{8}$ μM
C1q + $C1r_{(rec)}$, $C1s_{(rec)}$ ($1{,}1 \cdot 10^{-4}$ μM)	65,9 ± 5,7	1,05 ± 0,18	$5{,}0 \cdot 10^{5} \pm 0{,}9 \cdot 105$ μM

to occur stereospecifically within certain epidermal growth factor-like (EGF) modules of several mammalian proteins (Stenflo *et al.*, 1987; Prysiecki *et al.*, 1987). Both C1r and C1s has a potential site for this type of modification in the EGF domain (Asn 150 in C1r, Asn 134 in C1s). The analysis of the recombinant proteins showed, that 10% of recombinant C1r is hydroxylated (Monkovic *et al.*, 1992) while no β-hydroxy-Asn was found in recombinant C1s (Luo *et al.*, 1992).

For the first time an invertebrate α-ketoglutarate dependent hydroxylase activity was detected in Sf9 cells (Monkovic *et al.*, 1992). It was suggested by Thim *et al.* (1988) that the consensus sequence in the EGF module, alone is not enough for hydroxylase recognition. Hydroxylase seem to be very sensitive to steric factors. This might be the explanation for the decreased efficiency of insect cells in β-hydroxylation of the human EGF modules. Irrespective of the efficiency, these results establish the potential for aspartyl/asparaginyl hydroxylation in insects. Functional studies with the recombinant C1r and C1s revealed that β-hydroxylation in the EGF domain is not essential for the Ca^{2+}-dependent biological function of the C1 complex.

Expression of C1q

We made three pBlueBac transfer vectors for Clq expression containing the cDNA of the Clq A-, B- or C-chain (Dobó, 1993). The A- and C-chain was placed frompUC13(Petry et a, 1991;Petry *et al.*, 1992)into the transplacement vector. The B-chain was cut from the pAT153 plasmid (Petry *et al.*, 1989). The transfer vectors contain the following coding, 5'-noncoding and 3'-noncoding regions (in base pairs): 738, 29, 266 (A-chain); 762, 11, 148 (B-chain); 741, 38, 240 (C-chain). Recombinant viruses were then generated with these transfer vectors. Monolayer Sf9 cell cultures were infected simultaneously with all the three recombinant viruses (total multiplicity of infection was 9). We could detect all three C1q chains in the disrupted cells 3 days post infection but not in the cell culture supernatant. This might be the consequence either of the low number of effective triple infections or the deficient assembly of the chains since only the complete, processed C1q can be secreted and not the individual chains. To test this assumption the construction of a triple transfer vector expressing all the three chains of Clq in the same cell is in progress.

Expression of Cl-inhibitor

The cDNA of Cl-inhibitor was placed from pBlueScript plasmid (Bock *et al.*, 1986) into pBlueBac transfer vector. The clone contains 1503 bp coding region and 274 bp 3'noncoding region (the 35 bp 5'-noncoding region was excised). Recombinant viruses were then produced. The protein was secreted into the cell culture medium as detected by ELISA. The production level was about 2 $\mu g/10^6$ cells in our preliminary experiments.

C1-inhibitor was expressed in COS cells too (Eldering *et al.*, 1988). The product appeared to be identical to the human plasma-derived protein. In the case of baculovirus-insect cell expression system at least the glycosylation pattern is expected to be different from that of the serum C1-inhibitor. It is already known, that the carbohydrates have no effect on the inhibitory function of Cl-inhibitor (Coutinho *et al.*, 1994). Experiments to purify the recombinant C1-inhibitor and reveal its functional properties are in progress.

Increasing the yield of expression

In order to get higher amount of recombinant protein we have introduced modifications into the standard expression procedure. At DNA level we made C1r cDNA constructions using different transfer vectors (pAcYMl, pVL941, pBlueBac) and the 5'-noncoding region of the cDNA was also removed. We could increase the yield from 2 to 5 $\mu g/10^6$ cells using vectors which have the complete polyhedrin noncoding region. However the removal of the 5'-noncoding region did not influence the yield of expression.

In cell cultures we applied various insect molting hormones to stimulate the protein synthesis. 20-hydroxyecdysone proved to be the most efficient, producing a three fold increase in the level of recombinant protein secreted into the medium. This effect was also confirmed by tracing the L-(^{35}S) methionine incorporation into the gene product. Makisterone was also effective in stimulation, while ecdysone proved to be ineffective (Sárvári *et al.,* 1990).

Although most expression work has thus far been conducted in cell cultures, we have also used larval production as useful alternative to expression in cell culture, for generating large quantities of recombinant proteins. Recombinant viruses were used to infect the larvae of two Lepidopteran species, Mamestra brassicae and Manduca sexta. We used different methods for infections and optimized the time of protein harvesting from the insects hymolymph. Larvae were infected with recombinant baculovirus in their last larval stage. Both injection and feeding with encapsulated viruses were efficient. The optimum timing of the infection is the 60th hour of the last larval stage. Recombinant protein was detected in the hemolymph. Maximum recombinant protein (C1r. C1s) level was obtained when harvested 24 hours after infection (Hajós, 1993). In this way we can produce large amounts of biologically active recombinant protein at significantly reduced cost.

Genetic engineering

The expression of intact C1r and C1s in biologically active form has opened the way for genetic engineering studies. Our strategy, to reveal the structure and mechanism of activation of C1 at the molecular level, is based on the combination of genetic, physical and functional studies. The modular structure of the two subcomponents and the high degree of homology between them offer the possibility to construct deletion and hybrid mutants in addition to the point ones. The scheme of our approach is shown in Figure 5. The domain deleted and chimeric mutants were all folded and secreted into the cell culture medium. Some biological functions were preserved, while others disappeared. The results of this genetic engineering study might elucidate the role of regulatory domains (I–V) in the specificity and activation of C1r and C1s, these complex regulatory serine-proteases (Závodszky *et al.*, 1993; Gal *et al.*, 1994).

Discussion

These studies have demonstrated that sophisticated zymogens of human origin can be expressed in substantial quantities in the baculovirus-insect cell system. The recombinant C1r and C1s polypeptide chains, both are folded to form six independent globular motifs separated by flexible chain stretches. Apparently the recombinant subunits expressed by the insect cells undergo all essential features of posttranslational processing and folding, resulting in biologically active multifunctional proteins, secreted into the medium (Gál *et al.*, 1989; Luo *et al.*, 1992). Contrary to the accepted idea that insect cells attach only high-mannose-type oligosaccharides to proteins, complex type oligosaccharides may occur on proteins produced in cells infected by recombinant baculovirus, depending on the timing of the infection (Davidson & Castellino, 1991). In the case of recombinant complement subunits processing may have yielded small, complex oligosaccharides. The incompletely processed N-linked carbohydrate chains lack, at least, the terminal sialic acid, this deficiency was however not reflected in the biological activity of C1r and C1s (Luo *et al.*, 1992).

The presence of α- ketoglutarate-dependenthydroxylase activity was recently discovered in Sf9 cells. Recombinant human C1r expressed in Sf9 cells was found to be hydroxylated at the expected asparaginyl residue (Monkovic *et al.*, 1992), while recombinant C1s was not (Luo *et al.*, 1992). These observations point to the ability of insect cells to perform β-hydroxylation of Asp-containing EGF modules. The relatively low reactivity indicates the significance of steric requirements for substrate recognition.

The treatment of the infected cell cultures with various molting hormones can be used to increase the level of the recombinant protein synthesis (this might be the consequence of the increased mRNA level). The

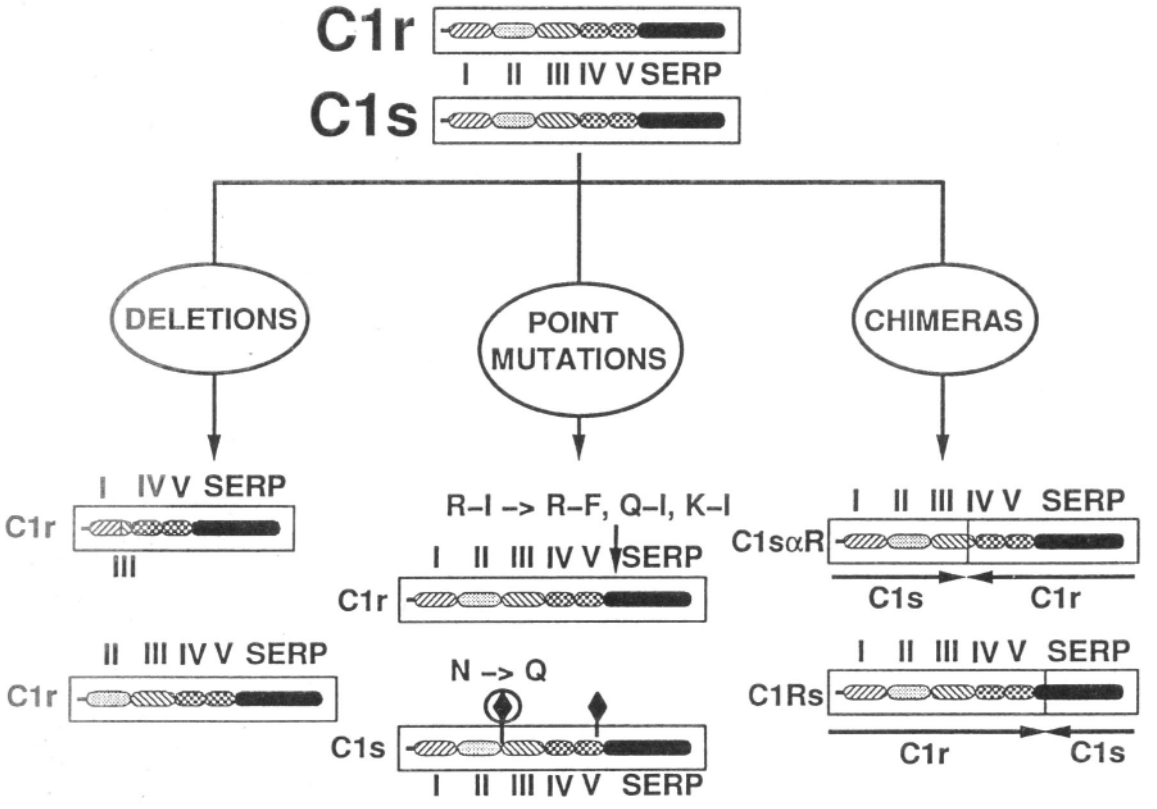

Figure 5. The strategy of mutation experiments. Diamonds indicate the Asn-linked carbohydrates of C1s. The arrow shows the Arg-Ile bond which is cleaved under activation.

suppression of the protein degradation machinery of the cells (e.g. the ubiquitin pathway) can be an other approach to enhance the yield. The selection or development of promoters, which can be switched on at various time points after infection, and the optimization of the timing of infection itself can be used to influence the yield of recombinant protein production, and the pattern of glycosylation.

The amount of recombinant protein can be increased in suspension cultures and the use of serum free medium during the last stage of the process could ease the purification of the recombinant protein product.

In an attempt to reduce the cost of recombinant protein production Lepidopteran larvae were used as "living bioreactors". Biologically active complement components were secreted into the hemolymph, after infecting the larvae with recombinant virus.

The expression of intact C1r and C1s in biologically active form has opened the way for concerted genetic engineering studies to reveal the mechanism of C1 activation. The scheme of our approach is shown in Figure 5.

Expression of complex, folded, glycosylated and processed proteins in biologically active form is a complex problem. The first step is to find the appropriate host-vector system which can do the job. We believe the insect-baculovirus system is a good choice and this belief is based on the experimental evidence accumulated during the past few years and was summarized in this paper.

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References

Arlaud GJ, Colomb MG & Gagnon J (1987) A Functional model of the human C1 complex. Immunol. Today 8: 106–111.

Bock SC, Skriver K, Nielsen E, Thogersen HC, Wiman B, Donaldson VH, Eddy RL, Marriman J, Radziejewska E, Huber R, Shaws TB & Magnusson S (1986) Human C1 inhibitor: primary structure, cDNA cloning, and chromosomal localization. Biochemistry 25: 4292–4301.

Borsos T, Loos M, Chapuis RM, Medicus R & Isliker H (1980) A novel way of relating the structure of C1q to the hemolytic activity of the first component of complement. Mol. Immunol. 17: 1415–1421.

Coutinho M, Aulak KS & Davis AE (1994) Functional analysis of the serpin domain of C1-inhibitor. J. Immunol. 154: 3648–3654.

Davidson DJ & Castellino FJ (1991) Asparagine-linked oligosaccharidc processing in Lepidopteran insect cells. Temporal dependence of the nature of the ologosaccharides assembled on Asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected Spodoptera frugiperda (IPLB-SF-21AE) cells. Biochemistry 30: 6167–6174.

Dobó J (1993) Diploma thesis. Institute of Enzymology, Budapest.

Donaldson VH & Evans RR (1963) A biochemical abnormality in hereditary angioneurotic edema: absence of serum inhibitor of Cl-esterase, Am. J. Med. 35: 37–44.

Eldering E, Nuijens JH & Hack C (1988) Expression of functional human C1 inhibitor in COS cells. J. Biol. Chem. 263: 11776–11779.

Gál P, Sárvári M, Szilágyi K, Závodszky P & Schumaker VN (1989) Expression of hemolytically active human complement component C1r proenzyme in insect cells using a baculovirus vector. Complement and Inflammation. 6: 433–441.

Gál P, Cseh S, Schumaker VN & Zádvodszky P (1994) The structure and function of the first component of complement: genetic engineering approach. Acta Microbiol. Immunol. Hung. 41: 361–380.

Hajós J (1993) Diploma thesis. Institute of Enzymology, Budapest.

Knobel HR, Villiger W & Isliker H (1975) Chemical analysis and electron microscopy studies of human Clq prepared by different methods. Eur. J. Immunol. 5: 78–82.

Kuroda K, Geyer H, Geyer R, Doerfler W & Klenk HD (1990) The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. Virology 174: 418–429.

Leytus SP, Kurachi K, Sakariassen S & Davie EW (1986) Nucleotide sequence of cDNA coding for human complement C1r. Biochemistry 25: 4855–4863.

Luo C, Thielens NM, Gagnon J, Gál P, Sárvári M, Tseng Y, Tosi M, Závodszky P, Arlaud GJ & Schumaker VN (1992) Recombinant human complement subcomponent C1s lacking β-hydroxyasparagine, sialic acid, and one of its two carbohydrate chain still reassembles with C1q and C1r to form a functional complex. Biochemistry 31: 4254–4262.

Mackinnon CM, Carter PE, Smyth SJ, Dunbar B & Fothergill JE (1987) Molecular cloning of cDNA for human complement component Cls. The complete amino acid sequence. Eur. J. Biochem. 169: 547–553.

Monkovic DD, VanDusen VJ, Petroski CJ, Garski VM, Sardana MK, Závodszky P, Stern AM & Friedman PA (1992) Invertebrate aspartyl/asparaginyl β-hydroxylase: potential modification of endogeneous epidermal growth factor-like modules. Biochem. Biophys. Res. Comm. 189: 233–241.

Patthy L (1994) Modular design of proteases of coagulation, fibrinolysis, and complement activation: implications for protein engineering and structure-function studies. In: Lorand L & Mann KG (ed.) Methods in Enzymology. Vol. 222 (pp. 10–22) Academic Press, New York.

Petry F, Reid KBM & Loos M (1989) Molecular cloning and characterization of the complementary DNA coding for the B-chain of murine Clq. FEBS Lett. 258: 89–93.

Petry F, Reid KBM & Loos M (1991) Gene expression of the A- and B-chain of mouse C1q in different tissue and the characterization of the recombinant A-chain. J. Immunol. 147: 3988–3993.

Petry F, Reid KBM & Loos M (1992) Isolation, sequence analysis and characterization of cDNA clones coding for the C chain of mouse C1q. Eur. J. Biochem. 209: 129–134.

Przysiecki CT, Staggers JR, Ramjit HG, Mussan DG, Stern AM, Bennett CD & Friedman PA (1987) Occurrence of β-hydroxylated asparagine residues in non-vitamin K dependent proteins containing epidermal growth factor-like domains. Proc. Natl. Acad. Sci. 84: 7856–7860.

Rapp HJ & Borsos T (1970) Molecular basis of complement action. Meredith Corporation, New York.

Reid KBM & Porter RR (1976) Subunit composition and structure of subcomponent C1q of the first component of human complement. Biochem J. 155: 19–23.

Sárvári M, Csikós Gy, Sass M, Gál P, Schumaker VN & Závodszky P (1990) Ecdysteroids increase the yield of recombinant protein produced in baculovirus insect cell expression system. Biochem. Biophys. Res. Comm. 167: 1154–1161.

Schumaker VN, Závodszky P & Poon P (1987) Activation of the first component of complement. Ann. Rev. Immunol. 5: 21–42.

Schumaker VN, Tseng Y, Poon PH, Bianchino AC & Závodszky P (1989) Spontaneous activation of reconstituted and serum Cl and the role of Cl-inhibitor. Behring Institute Mitteilungen 84: 102–110.

Sellar GC, Blake DJ & Reid KBM (1991) Characterization and organization of the genes encoding the A-, B- and C chains of human complement subcomponent Clq. Biochem. J. 274: 481-490./

Stenflo J, Lundwall A & Dahlback B (1987) β-Hydroxyasparagine in domains homologous to the epidermal growth factor precursor in vitamin K-dependent proteins. Proc. Natl. Acad. Sci. 84: 368–372.

Summers MD & Smith GE (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Tex. Agric. Exp. Stn., Bull., 1555, College Station, Texas.

Thim L, Bjoern S, Christensen M, Nicolaisen EM, Lund-Hansen T, Pedersen AH & Hedner U (1988) Amino acid sequence and posttranslational modifications of human factor $\mathbf{VII_a}$ from plasma and transfected baby hamster kidney cells. Biochemistry 27: 7785–7793.

Tosi M, Duponchel C, Meo T & Julie C (1987) Complete cDNA sequence of human complement C1s and close physical linkage of the homologous genes C1s and C1r. Biochemistry 26: 8516–8524.

Thielens NVM, Van Dorsselaer A, Gagnon J & Arlaud GJ (1990) Chemical and functional characterization of fragment of C1s containing the epidermal growth factor homology region. Biochemistry 14: 3570–3578.

Tschopp J, Villiger W, Fuchs H, Kilcherr E & Engel J (1980) Assembly of subcomponents C1r and C1s of the first component of complement: electron microscope and ultracentrifugal studies. Proc. Narl. Acas. Sci. 77: 7014–7019.

Tseng Y, Poon PH, Závodszky P & Schumaker VN (1991) Spontaneous activation of serum C1 in vitro. Role of C1 inhibitor. J. Immunol. 147: 1884–1890.

Vlak JM, Schlaeger E-J & Bernard AR (1992) Workshop on baculovirus and recombinant protein production processes. Editiones Roche, Basel.

Závodszky P, Gál P, Cseh S & Schumaker VN (1993) Protein engineering studies on C1r and C1s. Behring Inst. Res. Comm. 93: 103–114.

Ziccardi RJ (1982) Spontaneous activation of the first component of human complement (C1) by an intramolecular catalytic activity. J. Immunol. 128: 2500–2504.

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PART V
ECONOMIC AND REGULATORY ASPECTS

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Economics of baculovirus – insect cell production systems

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Key words: baculovirus, insect cell culture, economics, production, scale-up

Introduction

As the use of baculovirus – insect cell culture systems becomes established as a viable means of producing pharmaceutical proteins on an industrial scale, and as research and development investment in production of baculoviruses for agriculture increases, the question of the economics of production using these systems becomes more significant.

The use of baculovirus – insect cell culture systems for production of pharmaceutical proteins, including vaccines and diagnostics is discussed elsewhere in this issue, and is already considered proven technology in the industry, as is the use of mammalian cell cultures. In general, choice of a cell culture system for pharmaceutical production will depend on the characteristics required of the protein (in particular post-translational modifications), quality control issues, and productivity. Because of the relatively small amounts required, and the typically high price per unit treatment, production economics will have a lesser effect on the overall economics of the project than in the case of agriculture, which is characterised by application of large quantities over wide areas, at relatively low prices.

For this reason, this paper focuses primarily on the challenge of developing a cost-effective baculovirus product for agriculture, produced in insect cell culture. The model may be extrapolated for production of a recombinant protein by taking into account the productivity of the system, the processing cost of extracting and purifying the protein, additional quality control requirements, quantity of protein required per treatment, and anticipated price per treatment.

Production cost model

The production cost model of Bartholemew & Reisman (1979) has been used as the basis for constructing a model describing production of a baculovirus. Although numerous alternative models are available, and are widely used throughout the industry, the model used here has the advantage of describing in detail the various elements which are involved in developing a production system for a biological control agent used for insect control, in which biomass is harvested and applied in the field. An annual inflation rate of 5% has been assumed in updating costs. The model, therefore, is a valuable tool for illustrating the various factors which determine the economics of baculovirus production, and the effect which these factors, and interactions between them, have on the economics of the whole system. However, it should be considered as illustrative only; any real decision to invest in baculovirus production technology would be based on real costs, sales projections, interest rates, and production options, taking into account the fact that leasing and contracting arrangements may be preferred over capital purchases in many cases.

Assumptions used to construct the economic model

The assumptions upon which the basic model is constructed are listed in Table 1. All costs are quoted in US dollars ($).

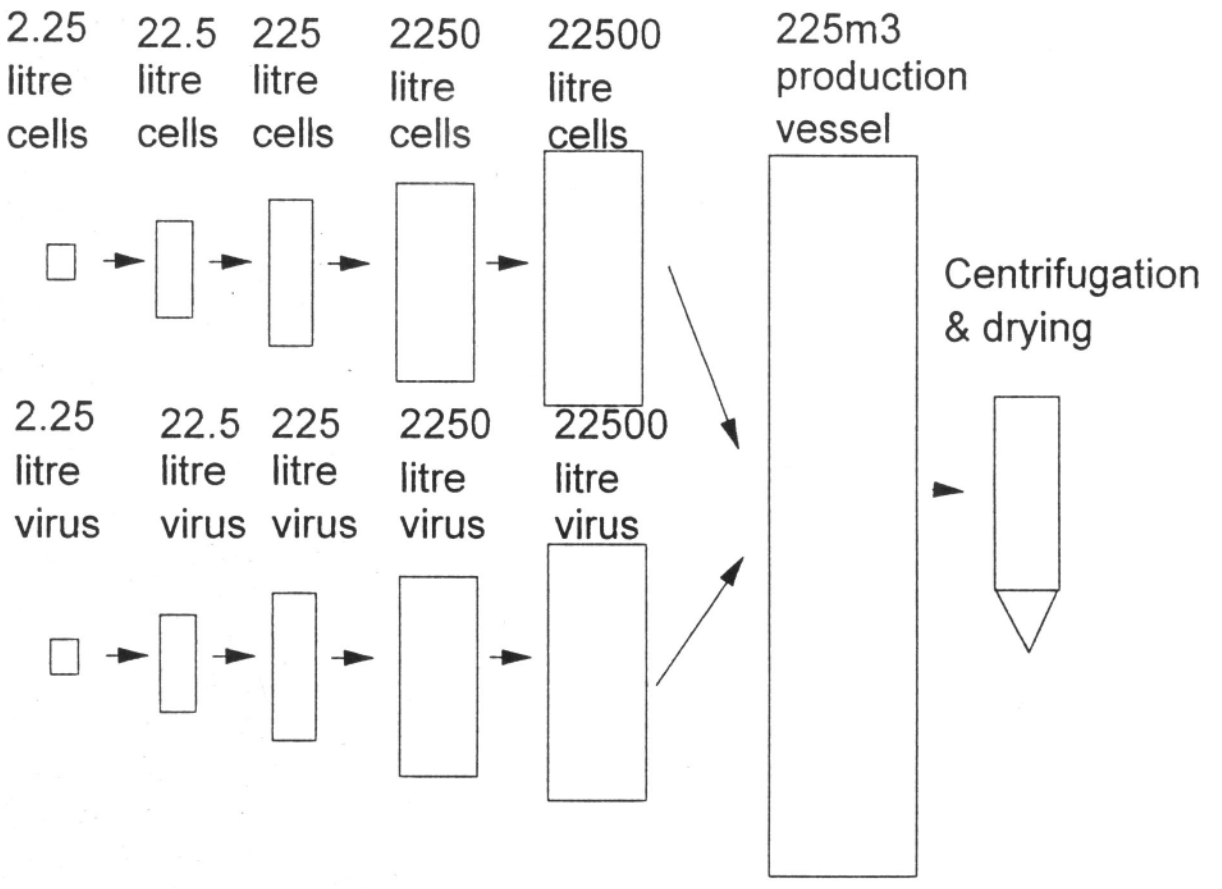

Figure 1. Production process.

Process assumptions

In constructing the basic model, a batchwise process has been assumed, in which cells are grown in a bioreactor vessel to a density of 4×10^9 cells l^{-1}, after which they are added, at a dilution of 0.1, to a tenfold larger vessel, and the process repeated until the production vessel is inoculated (Figure 1). Insect cell densities of between 2×10^9 and 5×10^9 cells l^{-1} have been obtained in fermenters (Zhang *et al.*, 1992; Maiorella *et al.*, 1988; Murhammer & Goochee, 1988; Klöppinger *et al.*, 1990; Ogonah *et al.*, 1991). The relatively low dilution rate is a result of the requirement of insect cells for high initial cell density in order to support continued growth (Goosen, 1991). Virus inoculum would be produced in the same way, by inoculating each seed fermenter with virus once an appropriate cell density was reached. The implication of this low dilution rate is that approximately 10 seed fermenters of varying size would be required to inoculate a production vessel of volume 225 m^3 (working volume 180 m^3). Increasing the number of seed fermenters will have a direct effect on both labour costs and the cost of control systems. The model assumes that conventional stirred tank reactors are used. Alternatives, for example airlift reactors, are possible (Agathos, 1991) and will differ with respect to capital cost, cycle time and energy costs. These factors must be taken into account in modelling the economics of the production system. The plant, consisting of four 225 m^3 fermenters, has been assumed to be operating at full capacity.

Downstream costs and losses are approximate only, and assume a biomass recovery step (centrifugation or filtration), followed by dehydration and incorporation into a wettable powder formulation. A loss of biomass of 10%, and a cost for downstream processing of 10% of the total production cost, has been assumed. Since a number of alternative approaches to processing and formulation of biological material are available (Rhodes, 1993), preliminary evaluation of the down-

Table 1. Assumptions used to construct the economic model

Volume of production fermenter	225 m^3
Working volume of production fermenter	180 m^3
Number of fermenters	4
Cycle time	9 days
Batches per year	162
Application rate	10^{12} PIB ha^{-1}
Cell density at harvest	4×10^9 cells l^{-1}
PIB density	100 PIB $cell^{-1}$
Production per year	1.17×10^{19} PIB
Area treated per year	11.7 Mha
Medium cost	\$1 l^{-1}
Utilities (Per 180 m^3 batch: Steam = 42.5 tonnes @ \$17.50 $tonne^{-1}$ + Cooling water = 8400 m^3 @ \$0.03 $(m^3)^{-1}$ + Electricity = 43200kWH @ \$0.065 kWH^{-1}) =	\$0.12 ha^{-1}
Capital cost of plant	\$45 million
Price of formulated virus (farmer level)	\$16.35 ha^{-1}
Formulation cost	\$1.00 ha^{-1}
Packaging cost (1 Kg ha^{-1} @ \$0.90/25 Kg bag)	\$0.04 ha^{-1}
Direct labour (8 employees × 4 shifts × \$34,900)	\$0.10 ha^{-1}
Indirect labour (proportion of direct labour)	40%
Salaried staff (proportion of direct labour)	85%
Associated costs (proportion of direct labour)	30%
Maintenance workforce costs (proportion of capital)	3%
Downstream processing losses	10%
Downstream processing cost (proportion of production cost)	10%
Depreciation period	12 years
Cost of capital per year	10%
Startup costs (proportion of capital)	10%
Cost of sales	12.5%

stream process, in order to define losses and costs more accurately, is an important step.

Cost, volume and price assumptions

Tramper & Vlak (1986) cite a field application rate of 10^{12} PIB ha^{-1}, and this has been incorporated into the model.

Costs may be classified as variable (those which vary with the volume of production) or fixed (those which are unaffected by the volume of production). These are listed in Table 2. Labour and associated overheads are considered to be fixed costs, since salaries of skilled workers typically are paid regardless of hours worked. Where several operations are undertaken in a plant, or where casual labour is used, a proportion of labour costs may be regarded as variable.

Table 2. Fixed and variable product costs

Fixed costs	Variable costs
Labour	Medium
Overheads	Formulation
Depreciation	Packaging
	Utilities

Cho *et al.* (1989) stated that the cost of insect growth medium had been reduced from \$90 l^{-1} to \$6 l^{-1} by eliminating fetal bovine serum and modifying the medium components. Goosen (1991) reported that a medium could be produced for \$6 l^{-1}, while Weiss *et al.* (1992) considered a medium cost of between \$3 and \$5 l^{-1} for production of baculoviruses to be achievable. On the basis of recent trends, and the expectation of continued improvements, therefore, a medium cost of \$1 per litre has been built into the basic model.

Formulation, packaging and utility costs, and price per hectare have been calculated on the basis given by Bartholemew & Reisman (1979) for a microbial insect control agent. The actual price per hectare which can be obtained will depend on the crop to which the baculovirus is applied, the technical efficacy of the product, and the availability of alternatives in the market. A detailed marketing study would be required in order to determine the price at which the product could be sold, and the effect which pricing would have on sales volume. Bartholemew & Reisman (1979) calculate net price on the basis of dealer discounts, distribution and freight costs at the rate of 30% of farmer level price, and this figure has been incorporated into the model. In practice, this assumption would need to be evaluated for each individual segment of the market.

The basic model assumes a product which has become well established in the market, with annual sales of \$191 m. No individual biological control product has yet achieved sales of this magnitude, although several chemical insecticides do so. Accurate prediction of sales volume is essential in order to evaluate the economic feasibility of the project, and to justify capital expenditure.

Productivity and cycle time assumptions

Baculoviruses have been reported to attain concentrations of 10 to 100 PIB per cell in bioreactors (Tramper *et al.*, 1990; Wang *et al.*, 1992; Klöppinger *et al.*,

Table 3. Profitability of baculovirus production in cell culture

	$ million per year
Sales	190
Discounts and transport	57
Sales ex manufacturer	133
Cost of goods	61
Gross profit	72
Cost of sales	16
Net profits (Year 2 to 10)	56
Startup costs	5
Net profits (Year 1)	51
Mean net profit (Years 1 to 10)	55
Return on sales 29%	

1990). Maiorella *et al.* (1988), and Klöppinger *et al.* (1990) infected cells after four days. Polyhedra were harvested by Tramper & Vlak (1986) two days post infection, and by Klöppinger *et al.* (1990) four days post infection. In the basic model, therefore, it has been assumed that the baculovirus reaches a density of 100 PIB per cell, with a cycle time of nine days, consisting of four days cell growth, three days virus multiplication, and two days for cleaning and re-sterilisation.

Economic analysis

Profitability

On the basis of the model described above, the total cost of production of a baculovirus in cell culture would be \$5.21 ha^{-1}, which compares very favourably with the \$19 ha^{-1} (excluding overhead, inflated at 5% p.a.) for *in vivo* production of *Heliothis zea* NPV quoted by Shapiro (1982), and would be profitable, resulting in a return on sales of 29% (Table 3).

Effect of cell and virus productivity

In comparison with a conventional bacterial fermentation, insect cells and baculoviruses multiply slowly and require expensive growth media. Fermentation costs therefore account for much of the total product cost. Consequently, productivity in the fermenter has a marked effect on the economics of production. This is illustrated in Figure 2. According to the model, production in cell culture becomes uneconomic below 1.7×10^{11} PIB l^{-1}. Given this sensitivity to virus productivity, a thorough assessment of virus productivity in suspension culture would be desirable before making an investment decision.

Effect of medium cost

Growth medium comprises a very significant proportion (48%) of the total cost of goods. The cost of growth medium therefore has a profound effect on the economics of production, as illustrated in Figure 3.

According to the model, a medium cost below \$2.50 would be required in order for the operation to be profitable.

Effect of application rate

A field application rate of 10^{12} PIB ha^{-1} has been assumed in the model. However, since viruses vary in virulence towards target species, the effective application rate may vary considerably. Moreover, a major constraint on the field application rate is the probability that a larva, feeding on the plant surface, will acquire an effective dose of the virus. More efficient formulations or application systems would be expected to reduce the application rate required to obtain a given level of control. Any variation in application rate will have a direct effect on the cost of goods.

Effect of productions cale

In order to examine the effect of varying production scale without introducing the complexity of varying capital cost, let us assume that the production plant is leased at a cost of \$100 000 per year, regardless of bioreactor volume, while retaining the assumption that the plant consists of four separate production vessels, and that the plant is producing at full capacity. It is evident (Figure 4) that profitability varies with bioreactor volume, the operation becoming profitable at a bioreactor working volume of approximately 11.5 m^3 (total working volume 46 m^3). Production on a pilot plant scale (working volume typically in the region of 5 m^3) is unlikely to be profitable unless fixed costs can be reduced substantially.

Continuous and batch production modes

Tramper & Vlak (1986) argued that a continuous process for production of baculovirus may be preferable on economic grounds to a batch process. In practice, it is not possible to produce baculoviruses in a truly continuous process, since repeated multipli-

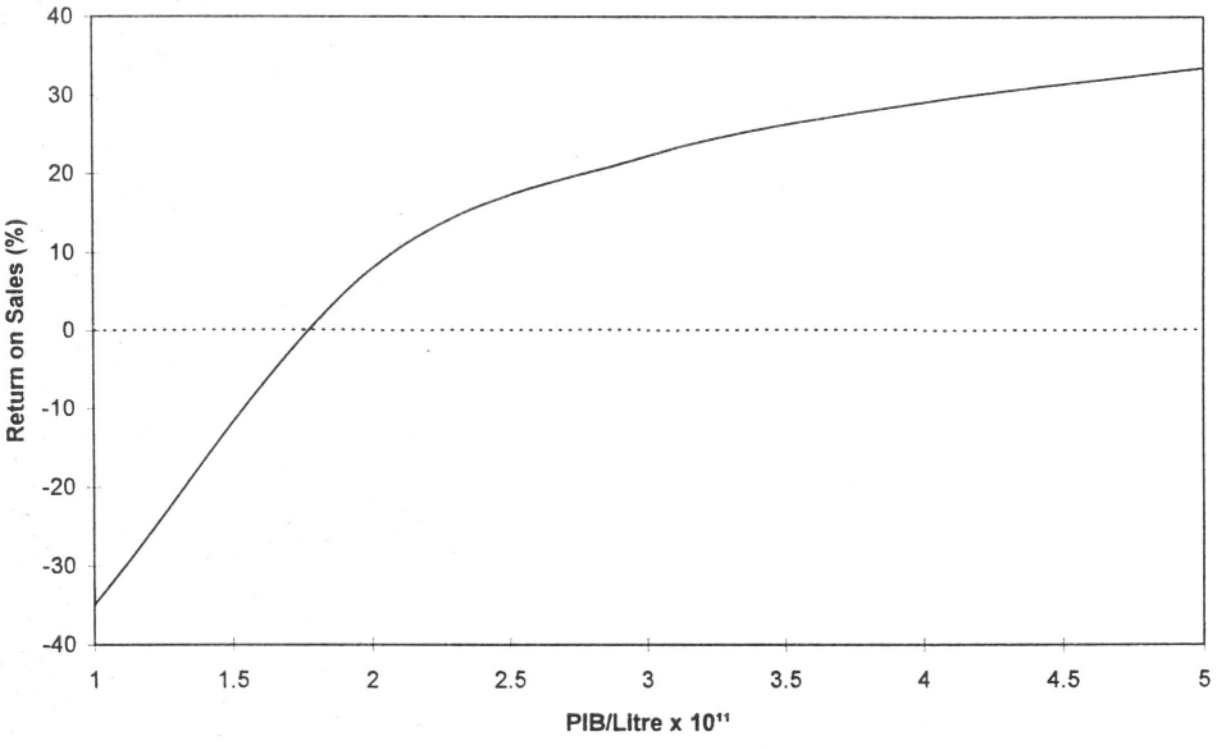

Figure 2. Effect of virus productivity on profitability.

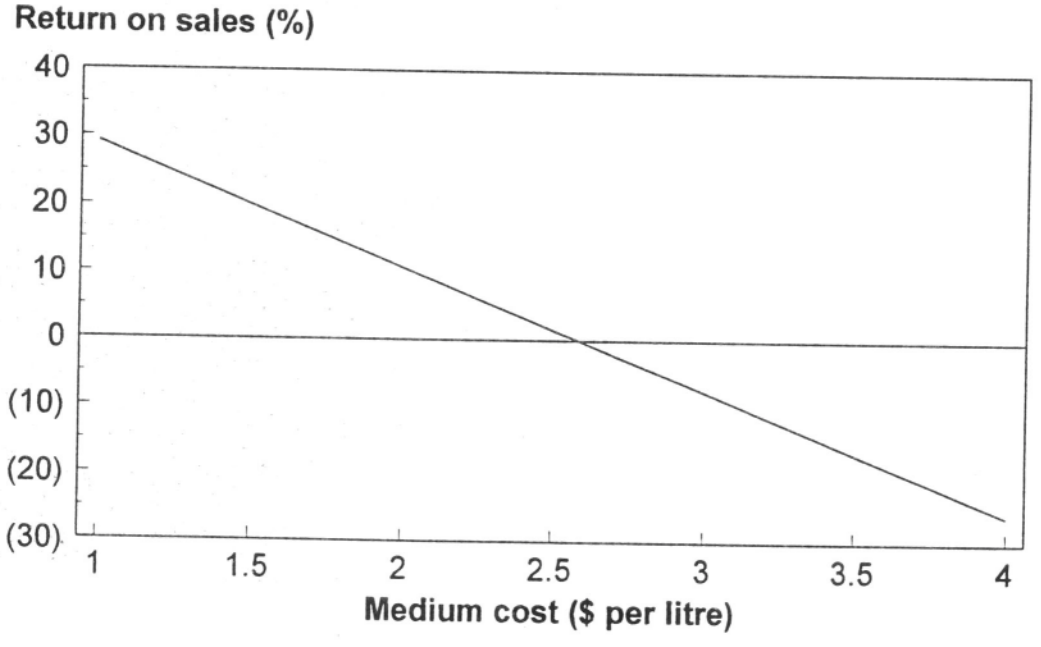

Figure 3. Effect of medium cost on profitability.

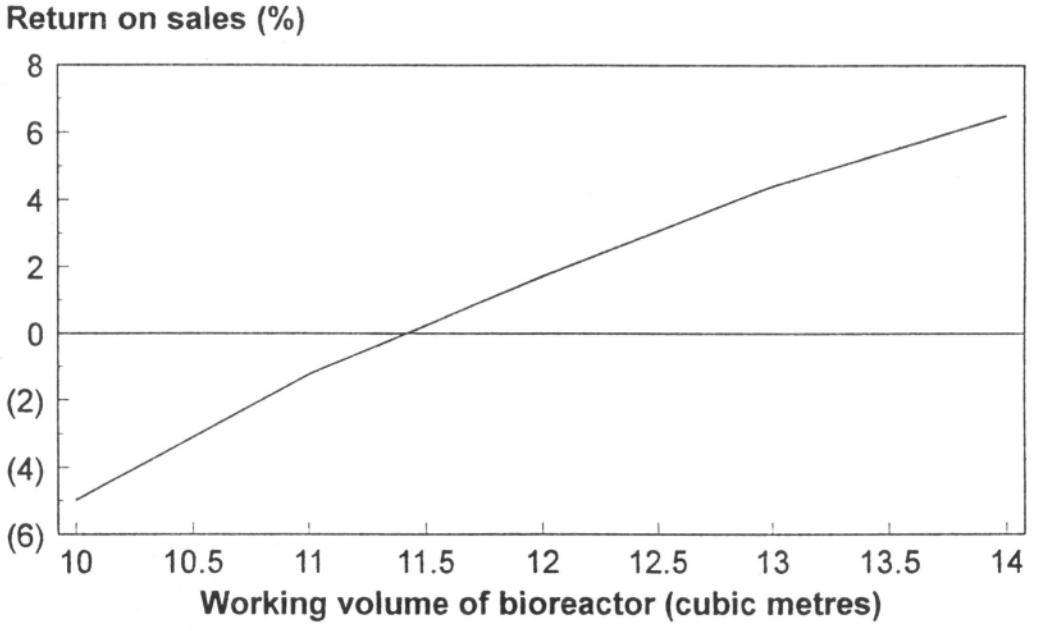

Figure 4. Effect of production scale on profitability.

cation in insect cells results in a loss of infectivity, as the proportion of defective interfering mutants in the population increases (Kool *et al.*, 1991). However, Tramper & Vlak (1986) indicated that continuous production could operate for up to a month before the passage effect would present a practical problem. The current model confirms that semi-continuous production, extending the production cycle from nine days to 30 days (including two days downtime in both cases) would have a beneficial effect on the economics of production, assuming that productivity of cells and virus is unaffected by production mode. A reduction in downtime from 80 to 24 days per year would increase annual output by 20%, thus reducing fixed production costs per hectare. The precise effect on profitability is difficult to quantify, since some variable costs (for example medium) will increase with increased output, whereas others (for example sterilisation of vessels) will not. The model indicates that continuous production would increase return on sales from 29 to 30%. However, the capital and startup cost of a continuous production system may well be higher than that of a batch system, and this would need to be offset against reduced production cost.

Project appraisal

Profitability (return on sales) is not the final determinant of whether a project involving capital expenditure is considered economically viable. Since money must be invested at an early stage of the project, before sales begin, the project must be evaluated in comparison with alternative investments. For this reason, a realistic projection of likely sales and profits over a period of several years is required, based on market research and the output of a model such as that described here. The project can then be compared with other alternatives on the basis of the return on investment (ROI). In this analysis, both production and product sales have been assumed to be at their maximum level. In practice, both would increase over time, and this gradual increase in sales and commitment of capital would be included in a more sophisticated model for the purpose of project appraisal.

Conclusions

Commercial production of pharmaceutical proteins in baculovirus – insect cell systems is already a reality, and has therefore not been discussed in detail here. Cost-efficacy will depend on the productivity

of the protein in culture, the dose, and the quantities required. According to the model described here, cost-effective production of baculoviruses for use in agriculture should also be feasible, assuming the commercial availability of a low-cost medium, together with a baculovirus with high productivity in cell culture, which is effective at a field application rate of 10^{12} PIB ha^{-1} or lower. All of these criteria appear to be achievable, given fairly modest advances over currently available technology. Given the relatively high fixed costs associated with production of baculoviruses on an agricultural scale in bioreactors however, profitability will depend on the scale of production. A substantial market opportunity (perhaps in the order of 1 million hectares) would be necessary in order to exploit the economies of scale achievable with baculovirus - insect cell production systems.

Acknowledgments

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References

Agathos SN (1991) Production scale insect cell culture. Biotech Advances 9: 51–68.

Bartholemew WH & Reisman HB (1979) Economics of fermentation processes. In: Peppier HJ & Perlman D (eds) Microbial Technology (pp. 463–496) Academic Press, New York.

Cho T, Shuler ML & Granados RR (1989) Current developments in new media and cell culture systems for the large-scale production of insect cells. Advances in Cell Culture 7: 261–277.

Goosen MFA (1991) Insect cell cultivation techniques for the production of high-valued products. Can. J. Chem. Eng. 69: 450–456.

Klöppinger M, Fertig G, Fraune E & Miltenburger HG (1990) Multistage production of *Autographa californica* nuclear polyhedrosis virus in insect cell cultures. Cytotechnology 4: 271–278.

Kool M, Voncken JW, van Lier FLJ, Tramper J & Vlak JM (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering particles. Virology 183: 739–746.

Maiorella B, Inlow D, Shauger A & Harano D (1988) Large-scale insect cell-culture for recombinant protein production. Bio/Technology 5: 1406–1410.

Murhammer DW & Goochee CF (1988) Scaleup of insect cell cultures: protective effects of Pluronic F–68. Bio/Technology 6: 1411–1418.

Ogonah O, Shuler ML & Granados RR (1991) Protein production (β-galactosidase) from a baculovirus vector in *Spodoptera frugiperda* and *Trichoplusia ni* cells in suspension culture. Biotechnol. Lett. 13: 265–270.

Rhodes DJ (1993) Formulation of biological control agents. In: Jones DG (ed) Exploitation of Microorganisms (pp. 411–439) Chapman & Hall, London.

Shapiro M (1982) In vivo mass production of insect viruses for use as pesticides. In Kurstak E (ed.) Microbial and Viral Pesticides (pp. 463–492) Marcel Dekker, New York.

Tramper J, van den End EJ, de Gooijer CD, Kompier R, van Lier FLJ, Usmany M & Vlak JM (1990) Production of baculoviruses in a continuous insect-cell culture. Ann. N. Y. Acad. Sci. 589: 423–430.

Tramper J & Vlak JM (1986) Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. Ann. N. Y. Acad. Sci. 469: 279–88.

Wang P, Granados RR & Shuler ML (1992) Studies on serum-free culture of insect cells for virus propagation and recombinant protein production. J. Invertebr. Pathol. 59: 46–53.

Weiss SA, Godwin GP, Whitford WG, Gorfien SF & Dougherty EM (1992) Viral pesticides: *in vitro* process development. Proc. 10th Australian Biotech. Conf.: 67–71.

Zhang J, Kalogerakis N, Behie LA & latrou K (1992) Investigation of reduced serum and serum-free media for the cultivation of insect cells (Bm5) and the production of baculovirus (BmNPV). Biotechnol. Bioeng. 40: 1165–1172.

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Safety aspects of insect cell culture

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Key words: biosafety, insect cells, containment, risk, virus, mycoplasma

Introduction

The generation of continuous cell lines from the tissues of insects has yielded valuable tools for biological studies. Some of these cell lines have also proved important hosts for expression of recombinant DNA. The primary concern with regard to safety in cell culture is that proliferating cells can provide a suitable medium in which some microorganisms, notably viruses, can multiply. However, safety considerations for insect cells cannot be dismissed due to the possibility that human pathogens might survive in insect cells. There are a number of levels at which the culture and manipulation of insect cells should be considered from the point of view of safe handling. In this overview we have attempted to outline the type of approach which should be adopted in risk assessment along with some practical suggestions to avoid problems and a brief summary of the current European safety standards.

Risk assessment

When using any new procedure or modifying an existing protocol it is essential to carry out a risk assessment of all aspects of the work process i.e. starting materials, culture procedures, product purification and waste disposal. For most chemicals and reagents used in cell culture there are standard texts and sources of information, most obviously the manufacturer, which enable rapid assessment of risk based on the properties of the reagent, its physical form, the quantities used and the procedures to which it is to be subjected. However, there are a number of factors unique to the manipulation and culture of animal cells which make risk assessment a more difficult and sometimes uncertain process. The following paragraphs aim to identify and give some general guidance in these key areas of difficulty with specific reference to insect cell culture.

1) Undefined components of growth media

Numerous growth enhancing compounds used in cell culture are derived from animal sources (e.g. human, bovine, mouse). Most significant of these is serum which, despite the development of serum free media, is a requirement in most cell culture work. Very often such supplements cannot be readily sterilised since this generally results in their inactivation or otherwise causes them to depreciate. Thus, being of animal origin, such materials represent potential sources of viral contamination and it is important to obtain them from suppliers which can guarantee that they stock only from uninfected sources or accredited virus free animal herds. Bovine serum has been implicated as the source of virus contamination of insect cell cultures and in some cases persistent infection has resulted (Hirumi, 1976; Plus *et al.*, 1980). Also in a recent report a group of viruses were detected in different batches of bovine serum (Erickson *et al.*, 1991). Some manufacturers may carry out tests for the detection of adventitious agents, but this is limited to specific organisms (e.g. mycoplasma and Bovine Viral Diarrhoea Virus). As a first step avoidance of human and primate sources of undefined reagents will minimize the risk of infection in laboratory workers. Some complex growth supplements and growth factors can be substituted with recombinant proteins, thus eliminating the risk of virus

transmission. However, such reagents are in general only available for mammalian cell culture.

The presence of adventitious agents which are not human pathogens should never be ignored due to the dramatic biochemical and genetic effects which they may have on cells in culture. Such infections could also disqualify a cell line, or its product, from patent or commercial licence applications. Fortunately for insect cell culture there are now a number of serum free defined culture media which offer a direct way to avoid the hazards of undefined components to the operator and cells in culture alike e.g. Sigma – serum free media 1 and 2, JRH Biosciences Ex-cell 401, Gibco Sf900/II. It is important to remember that the search for a cheaper culture medium may lead to the use of less pure reagents subjected to lower standards of quality assurance. Thus, in the long term the use of such reagents may be counterproductive both in terms of safety and quality of work.

2) Cells and adventitious agents

Risk assessment of animal cell cultures is a potentially confusing area as the cells are essentially undefinable and given to variation. The range of microorganisms which may be found in insect cell cultures has been reviewed by Vaughn, 1991. However, the primary cause for concern, in relation to laboratory safety, is the potential of cell cultures to sustain virus which may infect laboratory workers. Thus practical approaches to risk assessment of animal cell cultures have been based on the virological risk represented by the species and tissue of origin (Frommer *et al*., 1993; Stacey & Sheeley, 1991). Under these guidelines insect cells receive a very low rating in terms of risk due to the very low likelihood that the tissues of origin will harbour human pathogens. However, most arboviruses which replicate in insect vectors are also pathogenic to vertebrates and represent some serious human pathogens(e.g. Dengue fever virus, yellow fever virus). The risk which the cells of the vector insects can represent is exemplified by a report of carriage of certain haemorrhagic fever viruses in some mosquito cell lines (Ng *et al*., 1980). Such cases have been concluded to be the result of accidental laboratory contamination since the cell lines were established from non-feeding stages of the mosquito life-cycle (Vaughn, 1991).

Other types of virus may be present in insect tissues which can persist in primary cells (Vaughn, 1991). These viruses are generally non-pathogenic for humans although the possibility of infection risk cannot be excluded for a small number of insect virus groups (e.g. Entomoviruses, Cypoviruses and Iridoviruses). While it is obvious that the risk of zoonotic infections (e.g. arboviruses, pathogenic rickettsiae) in primary cells should be considered, insect cells collected from the environment may carry other human pathogens including brucella (Fotedar *et al*., 1991; Chadee & Le Maitre, 1990; Rady *et al*., 1992). Thus the risk assessment of primary cells and tissues should include consideration of the specific site and geographical location of origin.

Lack of dedicated facilities may lead to insect cells being handled in the same area as mammalian cells which provides a potential route for cross contamination with microorganisms present in mammalian cell lines. The organisms most likely to pass from mammalian cell lines to insect cultures and establish an infection are mycoplasmas. These organisms survive well in the environment, are unfortunately commonplace in animal cell cultures and can be extremely difficult to eradicate. It has been demonstrated that mycoplasma and acholeplasma can grow in insect cell lines and can establish persistent infection in drosophila cell lines (Hirumi *et al*., 1974; Hirumi *et al*., 1976; Steiner & McGarrity, 1983). Such infection can have dramatic effects on cells in culture, however, the species identified in cell culture are not generally associated with human disease except for *M. pneumoniae* (DelGiudice & Gardella, 1984). Thus, although the presence of these adventitious agents can have serious consequences for the infected cell line, they are unlikely to represent a serious health threat to laboratory workers provided that adequate containment and aseptic procedures, as required for good tissue culture technique, are observed.

3) Cell products

Natural cell products from animal cell cultures do not usually represent a hazard. Recombinant products, however, may demand higher priority in risk assessments. They should be assessed for their toxic properties, persistence in the environment and their ability to cause irritation or adverse immune reactions. It should be borne in mind that scale-up procedures will be aimed to produce high and concentrated yields of product which may represent significant hazards which were not evident at research and development stages. Accidental and incidental release of cell products into the laboratory environment should be provided for by adequate cleaning and decontamination procedures to be used as a matter of routine as well as in the event of

spillage. This will prevent the build up of cell culture products in the working environment.

4) Genetic modification and risk to the environment by recombinant insect virus vectors

Elsewhere in this volume the merits of insect specific baculoviruses as expression vectors of foreign genes are discussed. These viruses provide for the synthesis of very large quantities of recombinant protein in insect cell culture systems. One of the principal advantages of baculoviruses as expression vectors is their inherent safety for humans. The Baculoviridae represent a family of viruses which do not have members which have been isolated from mammals or any species other than arthropods. The use of other insect viruses which may represent a hazard (see above) and their use should be addressed in the risk assessment. However, this does not and should not preclude their use as expression vectors.

For the present, the major concern with the use of baculoviruses as expression vectors is the possible accidental release into the environment of a recombinant virus. Unmodified baculoviruses can persist in the environment for many years before encountering a susceptible host. This is attributable to the polyhedrin protein which surrounds the virus particles and protects them from physical abuse or ultra violet light. Fortuitously, most baculovirus expression vectors lack the native polyhedrin-negative virus, while fully replication competent and are thus unable to survive in the environment; this was tested in a planned field release experiment conducted in Oxford in 1987–1989 (Bishop *et al*., 1992).

Given that many users of the baculovirus expression system will be scaling-up virus-infected cell cultures to tens or hundreds of litres, the potential exists for a massive introduction of polyhedrin-negative virus into the environment. While the physical locations of fermentation plants should render interaction with susceptible insects most unlikely, there is a theoretical risk that some hosts could be infected. To counteract this remote possibility, the virus genome can be manipulated to remove genes encoding proteins required for replication in insects. An example is the AcNPV p74 protein, a component of the virus particle, which is required for virus uptake by insect gut tissue (Kuzio *et al*., 1989). Other modifications will undoubtedly become feasible, as we understand more about the complete sequence of the AcNPV genome.

The baculovirus gene promoters used to express most foreign gene products are active in what is known as the very late phase of virus gene expression. This phase appears to utilise an alpha-amanitin-resistant RNA polymerase which has yet to be fully characterized (Yang *et al*., 1991). However, the enzyme is very different to other RNA polymerases and experiments have determined that it is not active in mammalian cells. Hence, foreign genes under the control of the very late gene promoters of baculoviruses (e.g. polyhedrin) cannot be expressed in human cells and thus enhances the safety of laboratory manipulation.

It may be advantageous to limit the period of production of recombinant proteins to the scale up stage of cell culture. This would reduce operator exposure to hazardous proteins. Inducible gene expression systems, such as have been utilized in bacterial or mammalian-based vectors, have yet to be exploited in the baculovirus technology. This remains an area requiring further work.

5) Cell processing procedures

When all the various components of a particular process have been assessed individually it is important to then go over the proposed physical processes to be used and assess the level of containment required at each stage. Any procedures where aerosols are generated or materials may be accidentally transferred directly to an operators tissues and/or blood stream (e.g. use of hypodermic needles) should be reconsidered. In such cases it is important to identify alternative procedures or, if this is not possible, to ensure that reasonable precautions are taken to protect the operator and contain aerosols adequately. For procedures in which cells are to be lysed particular attention should also be paid to the potential release of naked DNA, especially from recombinant sources.

6) Decontamination procedures

It is important that the decontamination procedures are chosen specifically for each process. Selected disinfectants should be checked for their efficacy against those microorganisms likely to be present and the use of mixtures of disinfectants and/or cleaning agents should be checked for their compatibility. Decontamination procedures should be recorded as laboratory protocols with instructions for the preparation, use and regular replacement of 'in use dilutions' of disinfectants and cleaning agents. Cell culture contaminated materials

should be treated to ensure that they cannot carry any infectious particles when leaving the laboratory. In the case of heavily contaminated materials this is readily achieved by autoclaving. The mechanism of waste disposal should also be assessed to prevent outgoing waste contaminating new reagents and to prevent build up of waste which may then become a source of contamination within the laboratory.

Practical approaches to safe handling of insect cells

1) Use of safety cabinets

Since the detailed and cultural history of cell lines is often not known the possibility that they have become cross contaminated by viruses from other cultures cannot be excluded. Therefore all cell cultures should be handled within an appropriate microbiological safety cabinet. Where no human pathogen exceeding category 2 (see below) has been identified a Class II cabinet may be used. It is vital that this equipment is installed, monitored and maintained correctly (e.g. as given in the British Standard BS5726). In addition all staff involved in tissue culture work should receive training in the correct use of safety cabinets.

2) Organisation of work

Work practices should be designed to ensure that infected and new (i.e. uncharacterised) cultures do not contaminate culture media and other "clean" cultures. This can be readily achieved where there are separate areas for media preparation, "clean" (i.e. characterised) cell cultures and infected or uncharacterised cells. Where this is not the case work can be organised into sessions of increasing risk of contamination through each day followed by stringent decontamination. Such approaches, while important for safety, are also of scientific benefit in preventing the transmission of agents between cell lines.

3) Characterisation of cell cultures

In order for risk assessment of a cell culture procedure to be accurate it is important to confirm the authenticity of the cells in use. When dealing with cell lines with unique characteristics or primary cells isolated directly from tissue there is little chance that the wrong cells might be used. However, cell lines obtained from another laboratory, may have no proof of identity. Thus, it is necessary at least to have evidence of the species of origin of each cell line. It is also important to realise that the same cell line obtained from different sources can show phenotypic variation due to different culture histories (i.e. culture conditions and number of passages). In particular different clones of *S. podopterafrugiperda* cell lines are known to vary in their performance for expression of recombinant DNA.

4) Training

All laboratory staff should have training in good laboratory practice. For tissue and cell culture training in aseptic technique is also vital. Good aseptic technique although designed to prevent contamination of research material provides an effective barrier to infection of the operator. Specific training should also be provided in other aspects related to containment (e.g. disinfection, sterilization and fumigation) and any special hazards (e.g. cytotoxic drugs, genetically manipulated organisms) which may be encountered by each worker.

New directives on safety and biological agents

Following the directive from the European Parliament (EC Commission Directive 90/679/EEC) a unified system of classifying biological agents has been prepared with requirements and recommendations for containment of each class of agent. This classification of biological agents is based on that of the UK Advisory Committee on Dangerous Pathogens (ACDP) as are the requirements for containment of pathogens. As undefined complex biological systems, cell cultures represent uncertain hazards and as such should be treated as potentially infectious (i.e. as category 2 agents) even when an infectious agent has not been identified. In the case of insect cells the virus infections which might originate from the tissue of origin will not generally be significant in terms of the hazard to laboratory workers. However, certain adventitious agents, as indicated above, are a potential hazard to laboratory workers and it is therefore advisable to handle all cell cultures as if potentially infectious under containment level 2 conditions. Under European regulations these conditions include the use of an appropriate safety cabinet, spill resistant benching, documented disinfection procedures and a restricted access to the laboratory area.

Containment of cultures being transported between laboratories is extremely important as this represents a stage at which the general public and the environment

can become exposed to infectious organisms or environmentally hazardous agents. Regulations relating to the transportation of biological agents within Europe have been recently updated (e.g. CHIPS II 1994, UK; EC Commission Directive 92/1-3/EEC) and for global transportation by air IATA also have special regulations (DSM). Whatever the country of origin the person despatching cultures should remember that they have a responsibility to ensure that the recipient is aware of any hazards relating to each culture and that the recipient laboratory has appropriate facilities and staff to use the culture safely.

Conclusions

The hazards posed to the environment by the accidental release of baculovirus expression vectors can be put into perspective by the results obtained from experiments in which AcNPV was released deliberately into the field (Bishop *et al.*, 1992). Polyhedrin positive viruses will persist in soil and on leaf surfaces for periods comprising weeks and months. However, polyhedrin negative viruses (similar to those used as expression vectors) do not survive in similar situations. In consequence, accidental release of baculovirus expression vectors poses negligible hazard. The risk of such a release will largely depend on the skill of the operators. This does not take into account the hazard posed by the recombinant product which is being made by the virus-infected insect cell. Synthesis of a mammalian-specific toxin, of course, would require particularly careful manipulation of the virus-infected cell culture.

The fact that insect cell lines represent an undefined risk, in terms of carriage of adventitious agents means that their containment should be maintained at a minimum of the European containment level 2. Where the tissue of origin has a high risk of infection with human pathogens or where cells may have been used in a virus culture laboratory then appropriate testing is advisable. Careful risk assessment respecting the scale of work and whole procedures (in addition to individual assessment of agents and reagents) will ensure safe working conditions for laboratory staff. If applied properly safety procedures will also succeed in encouraging clean, efficient and well documented work procedures which are synonymous with the economical use of time and resources and good science.

References

ACDP (1990) Advisory committee on dangerous pathogens according to hazard and categories of containment. 2nd Edition. HMSO Books, London, UK.

Bishop DHL, Cory JS & Possee RD (1992) The use of genetically engineered virus insecticides to control insect pests. In: Release of genetically engineered and other organisms: 137–146. Day M & Fry JC (eds) Cambridge University Press, Cambridge, UK.

BS5726 British Standards Parts 1–4, HMSO Books, London, UK.

Chadee DD & Le Maitre (1990) Ants: Potential mechanical vectors of hospital infections in Trinidad. Trans. Roy. Soc. Trop. Med. Hyg. 84: 297.

CHIPS II. Chemicals (Hazards Information and Packaging for Supply) Regulations 1994. HSE books, Sudbury, UK.

DelGiudice RA & Gardella RS (1984) Mycoplasma infection of cell cultures: Effects, incidence and detection. In: *In vitro* Monograph 5. Uses and standardisation of vertebrate cell culture: 104–115. Tissue Culture Association, Gaithersburg, USA.

DSM Information on current international transportation and air-freight regulations for infectious organisms is available from the DSM, Mascheroder Weg 1b, D–38124 Braunschweig, Germany.

Erickson GA, Bolin SR & Landgraf JG (1991) Viral contamination of fetal bovine serum used for tissue culture: Risks and concerns. Dev. Biol. Stand. 75: 173–175.

Fotedar R, Shriniwas UB & Verma A (1991) Cockroaches (*Blattella germanica*) as carriers of microorganisms of medical importance in hospitals. Epidemiol. Infect. 107: 181–187.

Frommer W, Archer L & Boon B *et al.* (1993) Recommendations for safe work with animal and human cell cultures concerning potential human pathogens: *safe biotechnology (5).* Applied Microbiology and Biotechnology 39: 141–147.

Hirumi H (1974) Mycoplasma and viral contamination of insect cell cultures. Proceedings of the US. Japan cooperative conference on invertebrate tissue culture: Applications in fundamental research, Tokyo, Japan.

Hirumi H (1976) Viral, microbial and extrinsic cell contamination of insect cell cultures. In: Invertebrate Tissue Culture Research Applications: 233–268. K Maramorosch (ed.) Academic Press, New York, USA.

Kuzio JA, Jaques R & Faulkner P (1989) Identification of p74 a gene essential for virulence of baculovirus occlusion bodies. Virology 173: 759–763.

Ng ML & Westaway EG (1980) Establishment of persistent infections by flaviviruses in *Aedes albopictus* cells. In: Invertebrate systems *in vitro:* 389–402. Kurstak E, Maramorosch K & Dubendorfer A (eds) Elsevier/North Holland, Amsterdam.

Plus N (1980) Further studies on the origin of the endogenous viruses of *Drosophila melanogaster* cell lines. In: Invertebrate systems *in vitro:* 435–439. Kurstak E, Maramorosch K & Dubendorfer A (eds) Elsevier/North Holland, Amsterdam.

Rady MH, Abdel-Raouf N, Labib I & Merdan AI (1992) Bacterial contamination of the housefly, *Musca domestica,* collected from four Ciaro hospitals. J. Egypt. Soc. Parasitol. 22: 279–288.

Stacey GN & Sheeley HJ (1994) Have biosafety issues in cell culture been overlooked. J. Chem. Tech. Biotechnol. 61: 95–96.

Steiner T & McGarrity G (1983) Mycoplasma Infection of Insect cell cultures. *In vitro* 19: 672–682.

Vaughn JL (1991) Insect cells: Adventitious agents. Dev. Biol. Stand. 76: 319–324.

Yang CL, Stetler DA & Weaver (1991) Structural comparison of the *Autographa californica* nuclear polyhedrosis virus induced RNA polymerase and the three nuclear RNA polymerases from the host *Spodoptera frugiperda.* Virus Research 20: 251–264.

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Note: DSM information on current international transportations for infectious organisms is available from the DSM, Mascheroderweg 1b D38124, Braunschweig Germany.

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Regulatory issues in the use of insect-cell culture

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Introduction

Every manufacturer is confronted with regulations, which are aimed at minimizing the occurrence or the effect of possible risks associated with the production process. Several regulatory bodies are involved in risk assessment and control each having its own target group. Hence different regulations exist concerning protection of, for example, workers, the environment and the users of the product. Risks associated with the site of production affect primarily the staff operating the process, but can also have their impact on the environment. The environment does not consist of people alone and everything released into the environment (e.g. waste products, noise, (genetically modified) organisms) can have their influence on the ecological balance without having a direct hazardous effect on people. Also when assessing the risks of the application of a product, side effects with respect to the environment have to be taken into account.

Since the impact of risks is usually not restricted to one entity, more than one regulatory body may be issuing rules to minimize a certain risk. Regulations thus often overlap and it is often possible to comply to all regulations by applying the most stringent. However, there are always some points of conflict as Figure 1 illustrates. Sometimes these conflicts can be solved with a compromise (for example the dilemma of Figure 1 can be solved by applying down-flow air in a biohazard cabinet) or by giving one of the conflicting regulations priority after discussion with the authorities. This can, however, be difficult since no authority likes making a precedence. Another problem with complying to regulations is that regulations can differ from country to country and in some cases even within a country.

In another chapter (26) the biosafety of insect cells is discussed. This refers to risks of insect-cell culture for the workers and the environment and how to minimize these risks. This chapter will focus on the application of insect-cell cultures within the pharmaceutical industry. This industry has a long history of regulations and control. These regulations are very strict since the users of pharmaceutical products are a very vulnerable group. Most patients will have a lowered defence and since many drugs are injected several defence mechanisms of the body are surpassed anyway.

The legislation concerning drugs differs between countries and in principle for each country the manufacturer has to apply for a license. There are however co-operations between countries like the Pharmaceutical International Convention. The members of this convention accept results from inspectors from other member states. The European Community has issued directives which have to result in uniformity of drug laws within the EC.

Drug development

Despite different laws the development of a drug progresses via a standard route. The development of a drug is a time consuming process. It involves a long period of testing and along this period regulations are getting more stringent. In the preclinical phase of the research the efficacy and safety of the potential drug are tested in model systems. These are both *in vitro* and *in vivo* systems. Furthermore, a process is developed. The preclinical period usually takes 2–4 years. If the tested compound passes all tests and the authorities give permission it will enter the clinical phase. Testing of a product in the clinic involves three phases. In phase I the drug is tested on a limited number of people often

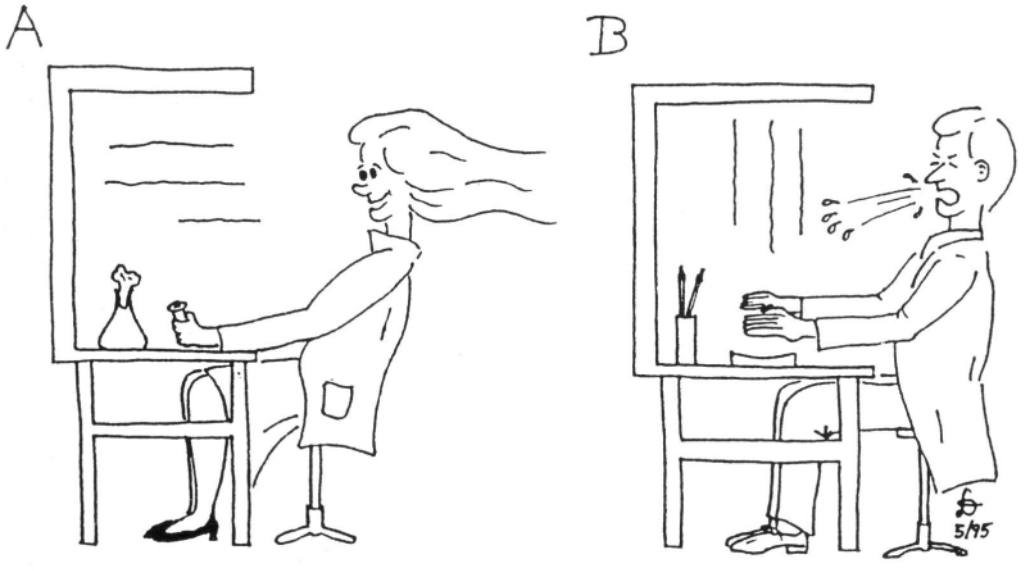

Figure 1. An example of conflicting protective measurements: a cross flow of laminar air will protect the product, but exposes the scientist to possible hazardous components (A), whereas a down flow of laminar air has the opposite effect (B).

not being patients. The purpose of clinical phase I is to test for possible side-effects. Phase II involves a large group of patients. In this phase the efficacy of the drug is tested and a dose level is determined. In phase III the production process is fully validated which involves production of 3–5 so-called consistency batches. Contaminant (viruses, DNA) removal studies are usually also performed during this phase. Material produced with the validated process is again tested in the clinic for long-term safety and efficacy. The testing in the clinic takes about 5–7 years. Based on the clinical tests an application is filed with the authorities. The preparing and review of this application can again take several years.

Since the testing of new drugs takes its time, production processes always are behind the current status of technology. Therefore it took some time before the first therapeutic product based on insect-cell technology entered clinical testing. This was the baculovirus-expressed gp160 envelope protein of human immunodeficiency virus (HIV) type 1. This possible AIDS vaccine appeared to be safe and immunogenic in volunteer patients with early HIV infection (Redfield *et al.,* 1991). The production of animal vaccines is discussed in other chapters of this issue (23, 22).

In the clinical phases of drug development (and of course in the final production for the market) the material is produced according to current good manufacturing practice (cGMP). The concept of GMP covers everything which has influence on product quality e.g. raw materials, processes, building, utilities, but also the company organisation. The whole concept of GMP can not be taken directly from a book. However, several regulatory bodies publish guidelines which, although they have no legislative status are considered as such by the industry. The most prominent of these are the "points to consider" published by the United States Food and Drugs Administration (FDA). Especially in biological processes there is a great diversity and the manufacturer has to state the specifications to be met. These specifications must not be too tight to allow for small culture differences, but on the other hand not too wide to prevent unacceptable product variations. This is one of the reasons manufacturers often fall back on "proven technology". Since one can fall back on existing and accepted data it is easier to get a process approved.

GMP production with insect cells

Prerequisite for all cGMP production processes is a building and an organisation which conforms with the GMP standards. For example there must be procedures in place to provide a hygienic environment (e.g. cleaning, gowning procedures), a proper quality control (of process, materials and environment) has to be in place, utility systems have to be validated and there must be a

system in place to maintain the level of quality (quality assurance). In this sense a process based on insect-cell culture will differ in no way from every other biological process. The first obvious difference is the cell material itself.

The basis of a GMP process is the master cell bank (MCB). The master cell bank is defined by the FDA (Points to consider 1993) as "a collection of cells of uniform composition derived from a single tissue or cell". The MCB is stored in liquid nitrogen and typically contains about 100 vials. A vial from the MCB is used to lay down a manufacturer's working cell bank or MWCB. This cell bank provides the starting material for the production of one production lot. For each new production lot a new MWCB vial will be thawed. The master cell bank has to be thoroughly tested on the absence of adventitious agents and on the identity. Testing on the MWCB is less severe since this bank is directly derived from the MCB. At the end of the production process another cell bank is laid down, referred to as extended or post-production cell bank (ECB, PPCB) or end-of-production cells (EPC). Often these cells are cultured a defined number of passages beyond the end of production before the final cell bank is made. Apart from the cell banks also intermediate products and of course the end product is subject to testing.

Health authorities are very reluctant to give lists of tests to be performed. These lists could easily become checklists which could result in ignoring differences between processes and state of the art knowledge of possible contaminations. Testing of cell lines with respect to adventitious agents should be based on an assessment of possible contaminants of the cell line. These contaminants could have been already present in the starting material or could have been introduced during the handling of the cell line (via media additions or operator handling). Therefore both mammalian and insect pathogenic viruses can be present in insect-cell cultures. The first being introduced for example via the introduction of serum in the culture medium. Since most mammalian viruses will not replicate in insect cells and most insect viruses not in mammalian cells, contamination levels of mammalian viruses will not become high and contamination with insect viruses will not be a great health thread. However, there are viruses capable of replicating in both cell types. The group of Arboviruses being the most prominent example although their insect host range is generally restricted to Dipteran insects (e.g. mosquito). Retroviruses are to date not detected in insect cells although the ability of baculoviruses to stably integrate host derived transposons is of some concern (McLean & Sheperd 1994). Apart from viruses the cell banks have to be tested for other possible contaminants like bacteria, fungi and mycoplasma's. In the "Points to consider" (1993) the FDA makes a separate reference to biological products made in insect cells: they should be tested for mycoplasma and spiroplasma contamination. It illustrates the inexperience with insect-cell processes that for mycoplasma testing suggested methods are available whereas the testing of spiroplasma should be discussed with the FDA. Biosafety testing is often done by specialised companies. In Table 1 a suggested biosafety testing schedule for insect-cell processes as published by one of these companies (McLean & Sheperd (1994) is given.

In contrast with other recombinant protein producing cells, the expression vector is usually not integrated in the insect cell. Since the most common way of modifying an insect cell is the use of a baculovirus expression vector, baculovirus stocks have to be created similar to the cell bank system. Since the virus stocks will be prepared via insect-cell culture, the same contaminants as present in insect cells can be expected.

Not only is it important to look for agents that should not be present, it is also important to know that what is present is like it should be. Already mentioned is the characterisation of the cell line. Morphology, growth characteristics and production levels should be stable throughout the production process. This last point raises some concern. Baculoviruses are not stable during serial passage through a cell culture. This phenomenon known as the passage effect is discussed in another chapter. Due to this passage effect production will decrease. This decrease is however reproducible. In Figure 2 the results of two continuous-production experiments are plotted. In one experiment insect cells, coming from a separate continuously operated bioreactor, are infected with wild-type baculovirus (Kompier *et al.*, 1988). In another experiment the cells are infected with a recombinant baculovirus expressing β-galactosidase (van Lier *et al.*, 1991). In both experiments production decreases after about four weeks. Furthermore in repeated batch experiments it has been proven that production of each batch reaches similar levels before the passage effect occurs (Zhang *et al.*, 1993, van Lier *et al.*, 1996). When the passage effect is properly characterized for a production process, it should be possible to define a limited number of batches to result in a consistent process.

Table 1. Safety testing schedule as proposed by Q-One Biotech (Technical bull. 12., McLean & Sheperd 1994)

Test	MCB[1]	MWCB[2]	PPCB[3]	Bulk harvest	Final product
Sterility	+	+	+	+	+
Mycoplasma	+	+	+	+	+
Quantitative transmission electron microscopy	+		+		
Reverse transcriptase assay	+		+		
In Vivo assay	+		+		
In Vitro assay	+		+	+	
number of detector cell lines	4–6		5–6	2–3	
Bovine virus detection assay	+			+	
Residual (insect) DNA					+
Pyrogenity					+

[1] Master Cell Bank.
[2] Manufacturer's Working Cell Bank.
[3] Post Production Cell Bank.

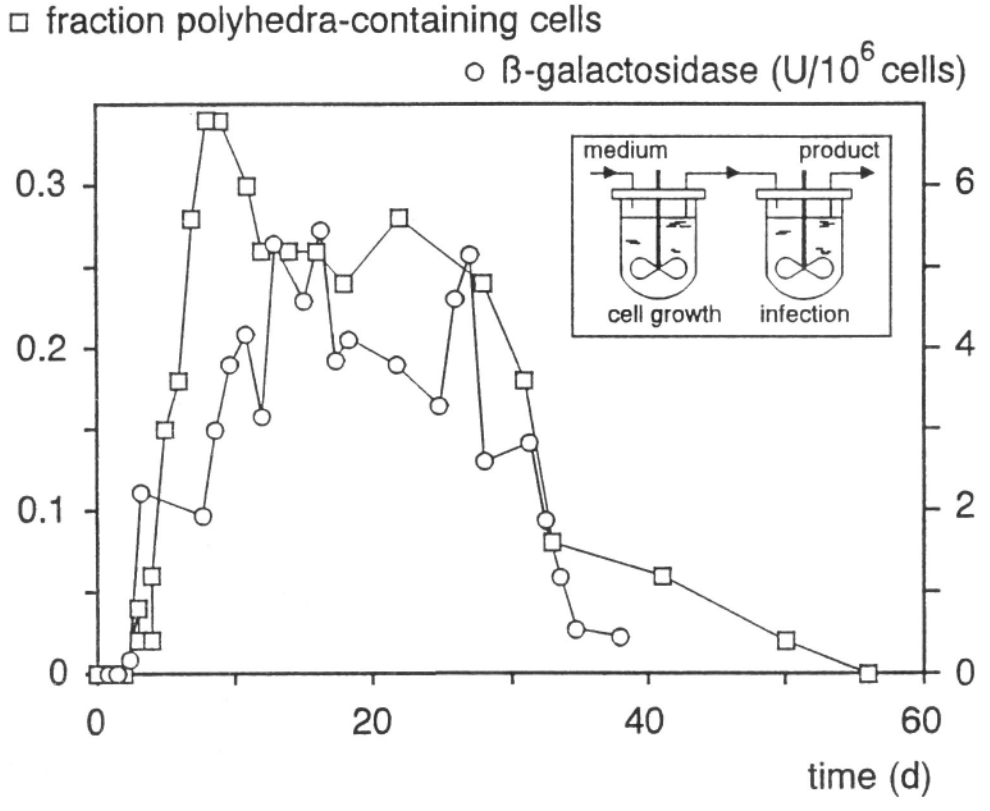

Figure 2. Production of polyhedra (□) and β-galactosidase (○) in a continuous bioreactor system (adapted from Kompier *et al.* (1988) and van Lier *et al.*, (1991) respectively). The insert schematically depicts the reactor configuration.

A hot topic raised in conjunction with insect cells and therapeutic proteins is post-translational modification or to be more precise glycosylation. Glycosylation in insect cells differs from that of animal cells. Complex-type oligosaccharides are usually not present on baculovirus-expressed proteins although the con-

trary is also observed (Davidson & Castellino, 1991). For a discussion on this topic the reader is referred to the relevant chapter in this issue (11). Since glycosylation can be crucial for proper functioning of the protein its effect has to be assessed. It has to be proved that a protein made in an insect-cell process is safe, effective and consistent. However, this is also the case for proteins expressed in other cells.

Conclusion

The insect cell as host for protein production is relative new. Therefore few data are available. This creates a vicious circle because it makes the choice of insect cells as basis for a pharmaceutical process less attractive. There are three main issues when comparing insect-cells to "traditional" systems as mammalian and bacterial cells. First, since the expression vector is not incorporated in the cells, a virus stock similar to the cell bank system has to be laid down and tested. This will cost time and money. Secondly the vector is subject to mutation and therefore the decrease in infectivity has to be characterized and validated. Third, the post-translational modification of the protein may differ. None of the mentioned issues, however, forms an obstacle that can not be overcome.

Acknowledgement

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References

Davidson JJ & Castellino FJ (1991) Asparagine-linked oligosaccharide processing in Lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on Asparagine–289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells. Biochem. 30: 6167–6174.

Kompier R, Tramper J & Vlak JM (1988) A continuous process for production of baculovirus using insect-cell cultures. Biotechnol. Letters 10: 849–854.

McLean C & Sheperd AJ (1994) The application of insect cells for biopharmaceutical production: implications for safety testing. In Spier RE, Griffiths JB & Berthold W (eds) Animal cell technology: Products of today, prospects of tomorrow, (pp. 769–774) Butterworth-Heinemann Ltd, Oxford.

Points to consider in the characterization of cell lines used to produce biologicals (1993). Center for Biologics Evaluation and Research, Food and Drugs Administration, Rockville.

Redfield RR, Birx DL, Ketter N, Tramont E, Polonis V, Davis D, Brundage JF, Smith G, Johnson S, Fowler A, Wierzba T, Schafferman A, Volvovitz F, Oster C & Burke DS (1991) A phase I evaluation of the safety and immunogenicity of vaccination with recombinant gp160 in patients with early human immunodeficiency virus infection. N. Eng. J. Med. 324: 1677–1684.

Van Lier FLJ, van der Meijs WCJ, Grobben NA, Olie RA, Vlak JM & Tramper J (1991) Continuous **β-galactosidase** production with a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. 22: 291–298.

Van Lier FLJ, van den Hombergh JPTW, de Gooijer CD, den Boer MM, Vlak JM & Tramper J (1996) Long-term semi-continuous production of recombinant baculovirus protein in a repeated (fed-) batch two-stage bioreactor system. Enzyme Microb. Technol. 18: 460–466.

Zhang J, Kalogerakis N, Behie LA & latrou K (1993) A two-stage bioreactor system for the production of recombinant proteins using a genetically engineered baculovirus/insect cell system. Biotechnol. Bioeng. 42: 357–366.

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13. Chaperone and foldase coexpression in the baculovirus-insect cell expression system

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18. Modelling baculovirus infection of insect cell culture

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19. Scale up aspects of sparged insect-cell bioreactors

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20. Oxygen gradiengs in small and big sparged insect cell reactors

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21. Down-stream processing of insect cell cultures

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Part IV: Applications

22. Parvovirus diagnostics and vaccine production in insect cells

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23. Classical swine fever virus diagnostics and vaccine production in insect cells

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24. Production of multidomain complement glycoproteins in insect cells

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Part V: Economic and regulatory aspects

25. Economics and baculovirus-insect cell production systems

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26. Safety aspects of insect culture

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27. Regulatory issues in the use of insect cell culture

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