

**ANIMAL CELL
TECHNOLOGY**
**Products of Today,
Prospects for Tomorrow**

Editors

R. E. Spier

Department of Microbiology, University of Surrey
Guildford, Surrey, UK

J. B. Griffiths

PHLS CAMR, Porton, Salisbury, Wilts, UK

W. Berthold

Dr Karl Thomae GmbH, Biberach an der Riss, Germany



EUROPEAN SOCIETY
FOR ANIMAL CELL TECHNOLOGY
THE 12th MEETING

BUTTERWORTH
HEINEMANN

CONTENTS

Section 1. Chairman's Statement

Presence and future of animal cell technology	3
---	---

Section 2. Making and Selecting Recombinant Cell Lines

Introduction of DNA into mammalian cells A J van der Eb	13
--	----

Retrotargeting: Use of defective retroviral DNA fragments to improve recombinant protein production in mammalian cells F M Wurm, A Johnson, T Etcheverry, Y S Lie, and C J Petropoulos	24
---	----

Receptor-mediated gene delivery into mammalian cells E Wagner, M Cotten, C Plank, K Mechtler, K Zatloukal and M L Birnstiel	30
--	----

Improvement of cell lines for large scale mammalian cell culturing K Kitano	35
--	----

Drosophila cell lines as hosts for recombinant protein expression C Cavegn, J Young, M Bertrand and A R Bernard	43
--	----

Immortalised cell lines for virus diagnosis J Clarke, C MacDonald, U Kreuzburg-Duffy and J B Griffiths	50
---	----

Selection strategies for highly productive recombinant cell lines H N Brand, S J Froud, H K Metcalfe, A O Onadipe, A Shaw and A J Westlake	55
---	----

Section 3. Cell Line Characterisation

Population analysis of a recombinant chinese hamster ovary cell line expressing recombinant human protein cultured in the presence and absence of methotrexate selective pressure M S Sinacore, S Brodeur, S Brennan, D Cohen, M Fallon and S R Adamson	63
--	----

Genetic and biochemical analysis of a murine hybridoma in long-term continuous culture A J Racher, G N Stacey, B J Bolton, A Doyle and J B Griffiths	69
---	----

DNA fingerprinting as a quality control marker for the genetic stability of production cells I Doherty, K T Smith and G M Lees	76
---	----

Analysis of SV40 early region expression in immortalized mouse macrophage cell lines U Ch K Kreuzburg-Duffy and C MacDonald	80
 Section 4. Complex Media Formulations	
Stimulation of murine hybridomas by cytokines J H Dempsey, D H Glass and F B Ward	85
The use of 2-hydroxy-2,4,6-cycloheptarin-1-one (tropolone) as a replacement for transferrin H Metcalfe, R P Field and S J Froud	88
Comparison of long R ³ IGF-1 with insulin in the support of cell growth and recombinant protein expression in CHO cells J N Thomas and V Fung	91
Development of a hybridoma cloning medium R Weedle, K Carroll, K Kavanagh, R O'Kennedy and M Clynes	96
Performance of gamma-irradiated fetal bovine serum in cell culture J D Keathley, D Wyatt, C M Williams, R Festen and C Maben	99
Low level FCS adaptation and media development for the culture of two hybridoma cell lines producing IgG and IgM monoclonal antibodies A Sanfeliu, B Damgaard, J J Cairó, C Casas, C Solà and F Gòdia	102
Improved bioreactor productivity and manufacturing efficiency using liquid medium concentrates D W Jayme, R M Fike, J M Kubiak and P J Price	105
Cell culture raw materials screening by calcein-AM fluorescence using a 96 well plate format T Ihrig, M Tsao, M Hilton, F Jacobson and M B Sliwkowski	110
Importance of extra-cellular matrix in the long term anchorage dependent growth of cell line CHO K1 M Bridges, C Gebert and P P Gray	115
 Section 5. Protein and or Serum Free Cultures	
Development of serum free media for engineered NSO cell lines C W Buser, R Beaudet, N Soohoo and G G Pugh	121
Chloesterol: An important additive for serum-free media? H Büntemeyer, M Burg and J Lehmann	124
Serum free ultraCULTURE™ medium: an alternative to the instability	127

generated by long-term cultivation of mouse hybridomas N Kessler, S Bertrand, G Thomas and M Aymard	
Investigations of the TSH antibody producing mouse hybridoma cell line under serum-free media condition E-J Schlaeger, T Klier and K Christensen	130
Interferon gamma production in laboratory bioreactor by <i>lens culinaris</i> lectin stimulated human buffy coat using protein free medium K Miomir, B Filipič, C Schmatz, K Carlsson, S Zupan, P Vrhovec, M Batič and B Kovač	134
Evaluation of CHO cell growth in a protein-free environment D E Wyatt	137
Inactivation of serum contaminants D E Wyatt, J Keathley, C Williams and R Broce	140
Adaption of mammalian cells to protein-free growth D E Wyatt	144

Section 6. Ammonia/Glutamine and Other Inhibitors

Ammonium ions inhibit cell growth by intracellular formation of UDP-activated hexosamines T Ryll and R Wagner	149
Influence of the pH on the ammonia sensitivity of a murine hybridoma cell line Lüdemann, R Pörtner and H Märkl	152
The influence of ammonium on t-PA production from chinese hamster ovary (CHO) cells C Dyring, H A Hansen and C Emborg	155
Detoxification of ammonium in bioreactor cultivations K Martinelle and L Häggström	158
Influence of lactate and ammonia on the death rate of hybridoma J L Goergen, A Marc and J M Engasser	161
The effect of glutamine-containing dipeptides on the growth and productivity of a hybridoma A Christie and M Butler	164
Inhibitory substance(s) secreted in cell culture media of recombinant CHO and a hybridoma cell line	167

S Siwora, M Fingberg, H Büntemeyer and J Lehmann	
Growth of hybridoma cells is inhibited by gangliosides H Brandt, J Müthing and J Lehmann	170
Section 7. Apoptosis and Cell Biology	
Programmed to die: Cell death via apoptosis T G Cotter	175
Cell death (apoptosis) in cell culture E-J Schlaeger and S D Simpson	183
Cell death by necrosis and adoptosis during the culture of commercially important cell lines R P Singh, M Al-Rubeai, C D Gregory and A N Emery	187
Spontaneous apoptosis contributes negatively to the regulation of hybridoma cell viability F Franěk	192
Metabolic characteristics and specific requests of hybridoma and CHO cell lines P Schorn, W Noé and W Berthold	199
Nature of cell-cell interactions between recombinant CHO cells expressing human interferon gamma S R Coppen, R Newsam, A J Baines and A T Bull	202
Section 8. Perfusion cultures and segregation methods	
High density insect cell culture for the production of recombinant proteins with the baculovirus expression system V Jäger, A Kobold, C Köhne, S M Deutschmann, E Grabenhorst, C Karger and H S Conradt	207
The super-spinner: A mass cell culture bioreactor for the CO ₂ incubator R Heidemann, U Riese, D Lükemeyer, H Büntemeyer and J Lehmann	212
Continuous suspension CHO cell culture H A Hansen and C Emborg	215
Progress in animal cell perfusion technology with superspin R Plüss, D Doyen, L van der Pol and J-L Romette	218
Production of a recombinant protein in a high density insect cell cytoflow reactor -X Deramoudt, S Monnet, J N Rabaud, J-M Qiuot, M Cerutti, G Devauchelle and M Kaczorek	222

A compact gravitational settling device for cell retention K J Thompson and J S Wilson	227
Conical bioreactor with internal lamella settler for perfusion culture of suspension cells C Knaack, G André and C Chavarie	230
Lamellar clarifier - a new device for animal cell retention in perfusion culture systems J Stevens, S Eickel and U Onken	234
Perfusion culture of suspended CHO cells employing inclined sedimentation J A Searles, P Todd and D S Kompala	240

Section 9. Fixed and Fluidized Beds

Collegenase production by a cell line in collagen microspheres in a fluidized-bed reactor and its modelization Th Marique, TH P D Blankaert, I Texeira Guerra and J Wérenne	245
Development of a bioreactor process for recombinant cell lines A J Racher and J B Griffiths	248
Growth and metabolism of CHO-cells in porous glass carriers E Lüllau, M Biselli and C Wandrey	252
Evolution of the capacitance of cytodex 3 microbeads suspended in growth medium V Degouys, F D Menozzi, D A Dubois, L Fabry and A O A Miller	256

Section 10. Porous microcarriers

High density culture using macroporous microcarrier J Shirokaze, M Nogawa and R Ogura	261
Growth of Spodoptera cells on porous microcarriers (Verax) production of recombinant proteins G R Pettman and C J Mannix	264
Protein free culture of R-CHO and hybridoma cells and macroporous polypore [®] carrier G Blüml, M Reiter, N Zach, T Gaida, C Schmatz, A Assadian, K Strutzenberger and H Katinger	267

Section 11. Hollow Fibre Bioreactors

A novel hepatocyte entrapment, hollow fiber bioreactor as a bio-artificial liver M V Peshwa, S L Nyberg, F J Wu, B Amiot, F B Cerra and W-S Hu	273
---	-----

Pitfalls of bioprocessing a human monoclonal multireactive IgM antibody 278
U Marx, D Roggenbuck, M Wilding, H Tanzmann and S Jahn

Hollow fiber cell culture - a proven alternative 281
B Horweth

Section 12. Novel Bioreactors

Development of a new membrane reactor for large scale mammalian cell culture 287
J L Goergen, A Marc, J M Engasser, J N Rabaud, G Pierry, V Geaugey,
I Geahel and J Hache

A simple magnetic driven mini-reactor for fluidized and packed beds 290
T Gaida, W Schich, M Reiter, G Blüml, N Zach and H Katinger

Animal cell cultivation in the NASA rotating-wall vessel 293
K C O'Connor, T L Prewett, T J Goodwin, K M Francis, A D Andrews
and G F Spaulding

Human cancer and primary cell culture in the new hybrid bioreactor 296
system tecnomouse
A Nagel, E Effenberger, S Koch, L Lübbe and U Marx

Analysis of a long term hybridoma culture in a new minimized high cell 299
density bioreactor
S Koch, H Tanzmann, Ch Riese, R v Baehr and U Marx

Cell-bubble interactions during aeration are strongly influenced by surfactants 302
in the medium and can be minimized in the newly developed bubble bed reactor
M Jordan, H Sucker, F Widmer and H M Eppenberger

Micro-bubble sparging of anchorage dependent animal cell cultures 305
A Handa-Corrigan, S Nikolay and R Brydges

Rationalization of the design of high density perfusion cultures for suspended 309
animal cells
A Handa-Corrigan, S Nikolay and S Zhang

Simulation of hybridoma growth and monoclonal antibody production in a 312
homogenous dialysis bioreactor
B Szperalski, F Geipel, T Lorenz, U Behrendt, J Wahl and M Comer

Section 13. Bioreactor Comparisons

Comparison of bioreactor technologies for efficient production of recombinant 315
 α -amidating enzyme
D E Matthews, K E Piparo, V H Burkett and C R Pray

A comparison of animal cell growth using microcarriers and suspended natural aggregates P M Alves, J L Moreira, A S Feliciano, M J T Carrondo	321
Comparative studies of cell propagation systems for production of human acetylcholinesterase by recombinant cells A Lazar, S Reuveny, C Kronman, B Velan and Shafferman	324
Production of a monoclonal antibody with a serum dependant hybridoma in different reactor systems H D Blasey, C O Isch and A R Bernard	329
Transient expression with COS cells on spinner scale H D Blasey and A R Bernard	331
Production of the HIV-1 neutralising human monoclonal antibody 2F5: Stirred tank versus fluidized bed culture M Reiter, A Buchacher, G Blüml, N Zach, W Steinfeldner, C Schmatz, T Gaida, A Assadian and H Katinger	333
 Section 14. Novel Measurements and Assays	
The cytosensor TM -microphysiometer: Biological applications of living cells on silicon sensor R Metzger	339
Mass spectroscopy determination of insect cell respiration rates in culture and production processes A A Kamen and R Tom	345
Intracellular pH evolution during batch cultures using flow cytometry M Cherlet, N Petitpain, M Dardenne, P Franck, J M Engasser and A Marc	351
Cell cycle analysis as a tool for control and regulation of mammalian cell cultures in bioreactors S M Deutschmann, U Valley, V Jäger and R Wagner	354
Use of MTT-assay in studies of the CHO: DG44 cell line M Hasenson	357
On-line-measurement of metabolites in mammalian cell bioreactors by means of enzyme electrodes and of the number of living and dead cells U Wellnitz and A Rakow	360
Fully automated image analysis of cell number, viability and morphology K G Tucker, J Welsh, A Al-Rubeai and C R Thomas	363
Fast quantification of mouse IgG from cell culture supernatants D Schell	366

On-line monitoring of growth and viral infection of VERO cells in collagen microspheres in a fluidized-bed bioreactor Th Marique, D Malarme, P Stragier and J W��renne	369
On-line glucose control of animal cell cultures in fluidized beds A P Loibner, N Zach, O Doblhoff-Dier, M Reiter, K Bayer and H Katinger	372
On-line indirect measurement of oxygen uptake rate in bench scale hybridoma cultures L K Nielsen and P F Greenfield	376
Software sensors for the monitoring of high-density hybridoma perfusion cultures F Pelletier, C Fonteix, A Louren��o da Silva, J L Goergen, A Marc and J M Engasser	379
Application of an industrial disc stack centrifuge for the separation of hybridoma cell as a first step in downstream processing R Kempken, A Prei��mann, J Sch��fer and W Berthold	383

Section 15. Physical factors in bioreactors

Dynamic interfacial tension and rheological properties of cell culture medium with shear protectant additives J Michaels, A K Mallik, J E Nowak, D T Wasan and E T Papoutsakis	389
Mixing phenomena in industrial bioreactors with perfusion spin filters K Jim Jem, S Fateen and J Michaels	392
Hydrodynamic control of suspended natural animal cell aggregates J L Moreira, A S Feliciano, P M Alves, J G Aunins, M J T Carrondo	397
Modelling and prediction of animal cell disruption in simple flows Z Zhang, C Born, M Al-Rubeai and C R Thomas	402
Biosynthetic responses and scanning electron microscopic studies of murine hybridomas subjected to hydrodynamic stresses S K W Oh, M Al-Rubeai, N Emery and A W Nienow	405

Section 16. Bioreactor operation strategies

Fed-batch strategies for mammalian cell cultures W Noe, P Schorn, R Bux, W Berthold	413
Growth and productivity of animal cells: A contribution to some factors influencing both G Kretzmer, A Ludwig, R Weidemann, M Teige, J Tomeczkowski	419
Simultaneous control of growth and productivity using a mutant CHO cell line	422

A Hovey, C Bebbington and N Jenkins

Factors affecting growth, infection and protein yield in baculovirus-infected insect cells N Kioukia, M Al-Rubeai, A W Nienow and A N Emery	425
Effects of the feed medium glucose concentration on growth and metabolism of hybridomas in chemostat culture J J Meijer, J P van Dijken and K Ch A M Luyben	428
Effects of glucose on the production of recombinant protein c in mammalian cell culture T Sugiura	431
Substrate level control in fed-batch hybridoma cultures B Romein, P van Londen, J J Meijer, C Hellinga, J P van Dijken and K Ch A M Luyben	434
Effect of dissolved oxygen on monoclonal antibody production from hybridoma cultured in haemodialysers G B Ryan, M T Simpson, W T Jones, M J Nicol, P H S Reynolds	437
Metabolic parameters of a hybridoma cell line in batch and continuous cultivation R Pörtner, A Bohmann, I Lüdemann, H Märkl	457
Overproduction of monoclonal antibodies in high salt medium in the presence of butyrate S K W Oh, P Vig, F Chua, W K Teo and M G S Yap	460
Growth control of production processes with BHK cells and regulation of the perfusion rate by monitoring the intracellular nucleotide pools U Valley, T Ryll and R Wagner	465
Control of the maximal cell density in a membrane perfusion reactor H Pinton, A Lourenço da Silva, J L Goergen, A Marc, J M Engasser, J N Rabaud and G Pierry	470
Continuous medium recycling in pilot scale with a recombinant animal cell culture for antithrombin III production U Riese, D Lütkemeyer, R Heidemann, H Büntemeyer and J Lehmann	476
Optimization of antibody production in a fluidized bed bioreactor G Rolef, M Biselli, R Dunker and C Wandrey	481
Monoclonal antibodies released from viable hybridoma cells at different stages of growth H Musielski, K Rüger, M Zwanzig, and K Lehmann	485
High cell density in insect cell cultures	494

S Reuveny, C W Kemp, J Shiloach

Semi-continuous production with the baculovirus expression system 504
F L H van Lier, M M Engelkes, L A Van der Pol, C D de Grooijer
and J Tramper

Long term cultures of BHK suspended aggregates 507
J L Moreira, A S Feliciano, J G Aunins, M J T Carrondo

Section 17. Kinetics and Modelling

Batch kinetic data of hybridoma growth and productivity as a basis for 513
simulation of antibody production in different culture systems
J Thömmes, M Biselli, M-R Kula and C Wandrey

Modelling insect cell cultures infected with recombinant baculovirus 518
L K Nielsen, J Power, K Radford, S Reid and P F Greenfield

Kinetic analysis of hybridoma growth and monoclonal antibody production in 521
semicontinuous and continuous cultures with respect to batch cultures
O-W Merten, D Moeurs, H Keller, M Leno, L Cabanie, E Couve

Process development and kinetic analysis of a protein-free hybridoma 532
perfusion system
S Mercille, M Johnson, L Bourget, R Lemieux and B Massie

Steady-state analysis of hybridomas in chemostat culture 539
J J Meijer, B Romein, J P van Dijken and K Ch A M Luyben

Kinetics studies of fed-batch hybridoma cultures: Effect of various feeding 542
compositions and flow rates
M Dardenne, M Cherlet, J M Engasser, A Marc

A general two-step procedure for the kinetic modelling of animal cell cultures 545
V Chotteau and G Bastin

Mathematical modeling of the growth and production kinetics in hybridoma cultures 548
R K Biener, R King, M Howaldt, W Ncé and E D Gillies

Section 18. Downstream processing

Impact of improved chromatographic media on productivity and process design in 553
downstream processing
C Schmidt, J H Berlöf and L-O Lindquist

Direct capture of recombinant proteins from animal cell culture using a fluidized bed 557
J C Erickson, J D Finch and D C Greene

Chromatographic separation of immunoglobins on a new "tentacle"-type "TA"-phase: Fractogel EMD TA 650(S) W Brümmer, E Müller and W Müller	563
Validation of chromatographic procedures used in the fractionation of plasma derivatives A J Darling, U Ch K Kreuzburg-Duffy, L Borland, A E Morrow, J H Berglöf and I Andersson	567
Section 19. Products from Animal Cells in Culture	
Successful products and future business prospects R G Werner	573
Production of recombinant factor VIII from perfusion cultures: I Large-scale fermentation B G D Bodeker, R Newcomb, P Yuan, A Braufman, W Kelsey	580
Production of recombinant factor VIII from perfusion cultures: II Large-scale purification B G D Bodeker, R Newcomb, P Yuan, A Braufman, W Kelsey	584
Advantages of high cell density in vitro cultures in down stream processing of a monoclonal IgG antibody for in vivo use P Preikschat, U Marx and R v Baehr	591
Production, purification and application of flatfish interferon H Murakami, T Tamai, T Noguchi, N Sato, S Kimura and S Shirahata	594
Monoclonal antibody production strategy for serum dependent clone C Isch, A R Bernard and H D Blasey	603
High level measles virus N protein expression using a recombinant baculovirus: Assembly of nucleocapsid-like structures A R Fooks, J R Stephenson, A Warnes, B Dowsett, B K Rima and G W Wilkinson	605
Equimolar expression of two protein chains in recombinant mammalian cells W Dirks and H Hauser	610
Expression of mammalian protein farnesyltransferase in a baculovirus system W-J Chen, J F Moomaw, L Overton, T A Kost and P J Casey	617
Levels of troponin I and myosin heavy chain in dedifferentiated adult rabbit cardiac myocytes I McPhee, K A Kane, C MacDonald	619
Expression of the D ₂ and D ₃ dopamine receptors in insect cells using the baculovirus system C Woodcock, P G Strange and B C Rooney	622

Production and characterization of soluble mouse and human interferon- γ receptors (IFN- γ R) from insect cells G Schmid, N Wild, M Fountoulakis, H Gallati, R Gentz, L Ozmen and G Garotta	625
Generation of DNA-packaging proteins by overexpression in the baculovirus/insect cell system V Sandig, J Forstova, N Krauzewicz, B E Griffin and M Strauss	633
Metalloproteinases and TIMPs production by aids-kaposi sarcoma derived cells D Blankaert, D Parent, C M Farber, J P Van Vooren, C Liesnard and J W�renne	638
Endothelial cells and their infection with the rickettsia Cowdria ruminant: prospects for vaccine production Ph Tott�, D Blankaert, T Marique, N Vachiery, I Guerra Teixeira, C Kirkpatrick, J P Vanvooren and J W�renne	643
 Section 20. Post translational modifications	
Changes in the glycosylation pattern of recombinant proteins effected by defined culture conditions of BHK-21 cells M Gawlitzek, C Villers, A Verbert, R Wagner, H S Conradt	649
Glycosylation analysis of a human monoclonal IgG antibody derived from a human-mouse heterihybridoma H Leibiger, A Hansen, H B�hme, U Marx	652
Culture pH and ammonia affect expression rates and glycosation of recombinant mouse placental lactogen proteins by CHO cells E T Papoutsakis, D I H Linzer and M C Borys	658
Analysis of the product consistency: independant of process parameters, rt-PA shows a stable glycosylation pattern K Kopp, W No�, Michael Sch�lter, F Walz and R Werner	661
Galactosylation of human/mouse IGG anti-NP antibodies and its detection by ELISA M Goodall, T Bentley, J Lund, N Takahasi and R Jeffries	667
Characterisation of carbohydrate chain on light chain of human monoclonal antibody H Murakami and H Tachibana	670
Comparison of the posttranslational modifications of human proteins secreted from different recombinant baculovirus infected insect cell lines E Grabenhorst, A Gr�ner, Volker J�ger, Marcus Ackermann, C Karge, K Rollwage, M Nimtz and H S Conradt	676
Characterisation of intra- and extracellular proteases in recombinant mammalian and hybridoma cells R B Kratje, W Lind and R Wagner	679

Impact of factors secreted by mammalian and insect cells on human monoclonal antibody integrity 683
M Ackermann, U Marx and V Jäger

BiP - a chaperone involved in antibody formation 688
I G Haas, C R Kaloff, M R Knittler

Section 21. Cells in culture for toxicology studies

Cell systems for *in vitro* toxicology 697
H G Miltenburger

Hepatocytes: *in vitro* pharmacology, toxicology and drug metabolism 711
J V Castell, M J Gómez-Lechón and M T Donato

Development of an optimised *in vitro* system for measurement of human tumour response to cytotoxic agents 723
C Mothersill, M Sheridan, J Harney, J Bonnar, T P Hennessy, C B Seymour

Three dimensional cell cultures for toxicity testing and for the estimation of the affinity of cells to biocompatible materials 729
U Wellnitz, A Rakow

Stable expression of the bovine adrenal 11 β -hydroxylase, a cytochrome P450 enzyme, in CHO cells as tool for drug characterization 732
T Petri and M Husemann

Activation and expansion of human cytotoxic T lymphocytes in hollow fiber bioreactors 735
C H J Lamers, R J van de Grind, J W Gratama and R L H Bolhuis

Section 22. Product Safety and Consistency Testing

Significance of contamination with viruses of cell lines used in the production of biological medicinal products 741
P D Minor

Residual *in vitro* activity of DNA in biologicals treated by beta-propiolactone: rabies vaccines as model 751
S Morgeaux, N Tordo, O Merten and P Perrin

The development of appropriate viral models for the validation of viral inactivation procedures 754
C Z-Tao, R Cameron, C Harbour and J P Barford

Regulatory affairs: Safety testing for gene therapy products 757

E Morgan and J Ostrove

Product quality studies of antithrombin III during medium recycling in pilot scale 760
D Lütkemeyer, U Riese, R Heidemann, M Bretschneider, J Lehmann, H S Conradt

Product consistency during long term fed-batch culture 763
D K Robinson, C P Chan, D K Lee, A B Lenny, T C Seamans, J-S Tung, P K Tsai,
J Irwin, C C Yu Ip, G E Mark and M Silberklang

The application of insect cells for biopharmaceutical production: Implications 769
for safety testing
C McLean and A J Sheperd

Generation of linear epitope specific antibodies against the human recombinant 775
tissue-plasminogen-activator (rt-PA)
F Hanakam, G Jung, W Berthold, R G Werner, W Werz

List of Participants 779

Author Index 816

Index 820

RECEPTOR-MEDIATED GENE DELIVERY INTO MAMMALIAN CELLS

Ernst Wagner, Matt Cotten, Christian Plank, Karl Mechtler, Kurt Zatloukal, and Max L. Birnstiel
Research Institute of Molecular Pathology, Vienna, Austria.

We have developed gene transfer systems which use the receptor-mediated endocytosis route to import DNA into mammalian cells. DNA gene constructs have been complexed with a polylysine-conjugated ligand (such as transferrin) for uptake via receptor-mediated endocytosis and polylysine-conjugated, endosome-disruption agents (such as replication-defective adenoviruses or peptides derived from the N-terminal sequence of influenza virus hemagglutinin) which allow cytoplasmic entry of the DNA. These complexes have been delivered to and expressed at very high level in a large proportion of target cells (up to 80% in primary fibroblasts, primary myoblasts or primary human melanoma cells).

Key words: DNA transfection; gene therapy; gene transfer; endocytosis; transferrin; polylysine; endosome disruption; adenovirus; influenza peptide

Introduction

Our work has been based on the idea of adopting cellular transport mechanisms for the uptake of nucleic acids into cells. In order to exploit the transferrin receptor mediated endocytosis, the iron transport protein transferrin that has been chemically conjugated to the DNA-binding polycation polylysine (1,2). Polylysine upon binding to the DNA also condenses it to a doughnut-like particle (3). The complexes have been found efficiently delivered into the endosomes of many types of cells. In some cell lines a high level gene expression has been found, in many cell types, however, low or no expression has been observed (4).

Results

It became clear that the DNA is accumulating in internal vesicles and subsequent steps that deliver the DNA to the nucleus work only inefficiently. Strategies had to be developed to prevent degradation of the DNA in lysosomal compartments and promote the transport to the nucleus. This has led to the development of an efficient gene transfer system (5) consisting of a transferrin-polylysine / DNA complex linked

to a replication-defective adenovirus (see **Figure**). After endocytosis of these combination complexes into endosomes the natural endosomal acidification process triggers a conformational change in adenoviral coat proteins which then destabilize the membranes; both the viral particle and the DNA are released to the cytoplasm.

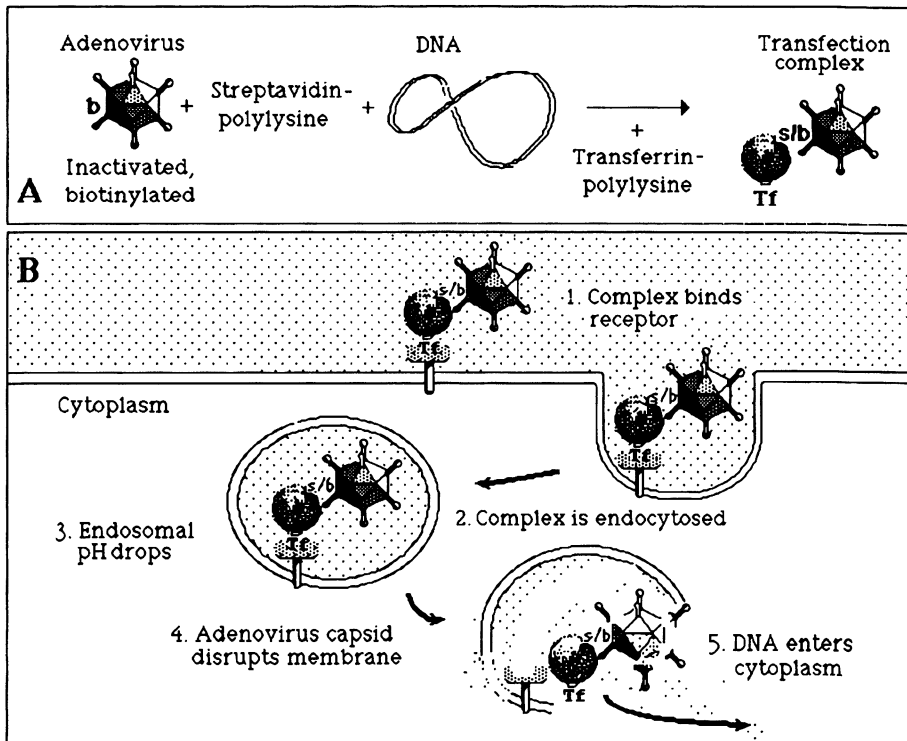


Figure: Formation (A) and uptake (B) of adenovirus-polylysine / transferrin-polylysine / DNA combination complexes into cells.

The use of replication-defective and chemically (psoralen /UV) inactivated adenovirus (6) avoids most of the potential hazards associated with viral vectors. The delivered gene is carried on the exterior of the adenovirus, being therefore far less restricted in size or sequence of the DNA to be delivered. Transfer of 48 kb DNA molecules, and high level expression of the full length (8 kb) human factor VIII cDNA in primary fibroblasts, myoblasts and myotubes has been demonstrated.

A potential application of the gene transfer system may be the development of a cytokine gene-modified melanoma vaccine. This is supposed for application in patients with minimum residual disease where most of the tumor mass has been removed by surgery. A tumor cell culture has to be established, transfected with a immunostimulatory agent such as a cytokine gene, irradiated with 100 Gy in order to block replication. Such a cytokine-gene modified irradiated vaccine when injected s.c. into the patient is supposed to induce a systemic immune response against residual tumor cells that otherwise lead to metastasis formation.

Cytokines are important actors in the activation of an immune response. Interleukin 2 (IL-2) is produced by stimulated T-helper cells that have seen e.g. tumor-antigen presented by cells such as macrophages or dendritic cells. IL-2 stimulates effector cells such as NK cells or CTLs that are able to circulate in the patients body and to eliminate residual tumor cells. IL-2 expressing tumor cells should have the ability to directly activate these effector cells.

We have started first animal experiments in the M3 melanoma model. Murine M3 melanoma cells transfected with cytokine gene constructs produce high levels of cytokines (24,000 units IL-2 / 10^6 cells/24 hrs; 1200 ng IFN- γ / 10^6 cells/24 hrs). Transplantation of 1×10^5 IL-2 transfected M3 cells into syngeneic DBA/2 mice showed loss of tumorigenicity (0/6 animals), whereas 6/6 animals treated with 1×10^5 untransfected M3 cells developed tumors. In order to test a biological efficacy of the cancer vaccine, animals were immunized with irradiated, IL-2 transfected M3 cells. The vaccination resulted in a systemic protection in all animals against subsequent challenge with tumorigenic doses of unmodified M3 cells (7).

Conclusions

What are the future options? On the one hand we want to further develop the cancer vaccine into a stage suitable for a clinical trial, on the other hand we are developing completely synthetic versions of the gene transfer system. We have replaced the whole virus by small synthetic peptides similar to sequences occurring in the hemagglutinin of influenza virus. At neutral pH this peptide have an inactive, rather unordered structure, whereas at acidic, endosomal pH they adopt the active structure of an amphipathic helix that can interact with the lipid membrane and destabilize it. We have incorporated such endosome-disruptive peptides into complexes containing DNA, transferrin and polylysine. The peptides within the complex are able to disrupt liposomes (8) or endosomal membranes (C.P. and E.W., unpublished results) triggered by the change to lower pH and substantially (up to 10,000-fold) augment the gene transfer (8,9).

References

- 1 Wagner, E., Zenke, M., Cotten, M., Beug, H. and Birnstiel, M.L. Transferrin-polycation conjugates as carriers for DNA uptake into cells. Proc. Natl. Acad. Sci. USA 1990, 87, 3410-3414.
- 2 Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H. and Birnstiel, M.L. DNA-binding transferrin conjugates as functional gene-delivery agents: synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety. Bioconjugate Chem. 1991, 2, 226-231.
- 3 Wagner, E., Cotten, M., Foisner, R. and Birnstiel, M.L. Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells. Proc. Natl. Acad. Sci. USA 1991, 88, 4255-4259.
- 4 Cotten, M., Wagner, E., and Birnstiel, M.L. Receptor mediated transport of DNA into eukariotic cells. Methods Enzymol. 1993, 217, 618-644.
- 5 Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D.T. and Birnstiel, M.L. Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. Proc. Natl. Acad. Sci. USA 1992, 89, 6099-6103.
- 6 Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D.T. and Birnstiel, M.L. High-efficiency receptor-mediated delivery of small and large (48kb) gene constructs using the endosome disruption activity of defective or chemically inactivated adenovirus particles. Proc. Natl. Acad. Sci. USA 1992, 89, 6094-6098.
- 7 Zatloukal, K., Schmidt, W., Cotten, M., Wagner, E., Stingl, G. and Birnstiel, M.L. Gene Therapy for Cancer: The Utility of Transferrinfection in generating "Tumor Vaccines". Gene 1993, in press.
- 8 Wagner, E., Plank, C., Zatloukal, K., Cotten, M. and Birnstiel, M.L. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine/DNA complexes: Towards a synthetic virus-like gene transfer vehicle. Proc. Natl. Acad. Sci. USA 1992, 89, 7934-7938.
- 9 Plank, C., Zatloukal, K., Cotten, M., Mechtler, K. and Wagner, E. Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. Bioconjugate Chem. 1992, 3, 533-539.

PAPER OF WAGNER

Spier: While recognising that the introduction of exogenous genes into animal cells is important have you examined the moral issues involved in the use of such techniques for the enhancement of human functions rather than the alleviation of disease ?

Wagner: The concept of our work is that it is not the permanent transfer of genes into cells. We chose the applications of the tumour vaccines because once it has worked there will not be any genetically modified cells remaining in the patient. The gene transfers we use lead to episomal DNA and not integrated DNA.