Cytotechnology **36:** 3–32, 2001.

Lysine: Is it worth more?

Debatosh Datta*, Akshay Bhinge & Vidya Chandran
School of BioMedical Engineering, Indian Institute of Technology-Bombay, Powai, Mumbai, India
(Author for correspondence, E-mail: ddatta@cc.iitb.ac.in)*

Received 4 April 2001; accepted 30 August 2001

Key words: biological properties, food preservative, immune therapy, lysine

Abstract

Lysine, an essential cationic amino acid, has a positively charged R group. The structure of lysine is given as (H_3N^+-) CH(-COO^-)-CH_2-CH_2-CH_2-CH_2-N^+H_3. While the anabolic role(s) of the molecule has been in focus for quite a few decades now, its biological properties, e.g. role in cellular proliferation *in vitro* (both anchorage dependent and anchorage independent) and *in vivo*, its ability to induce strong inflammatory and immune responses – both humoral and cell mediated, its role in augmented healing of all types of wounds in animal models as well as in human subjects (both acute and chronic), as well as its role in inducing extensive angiogenic responses, have never received reasonable attention so far. In the current brief and indicative review (rather than exhaustive reviews of each area), we intend to bring these biological properties of the molecule to focus while discussing a few other interesting aspects – lysine as a food preservative as well as its possible role(s) in immune therapy. While the areas look extremely divergent, we propose a common denominator in the form of a possible molecular mechanism of action of the molecule in all these diverse situations.

Lysine as a cell proliferator

Monomeric L-lysine HCl was supportive of cellular division and growth *in vitro* in an optimal concentration range of 7–10 μg ml^{-1} depending on the cell line (Datta et al., 1997a, b). We examined the phenomenon in few anchorage independent (AE9D6, CC9C10, HUT 78) and anchorage dependent (BHK 21 from NCCS) cell lines. Peak cellular growth was obtained, in all cases at 48 hr of culture (which could not be accounted for) and maximal cell concentration depended on a) initial cell density at seeding and b) cellular adaptability to culture conditions in terms of number of passages it went through before lysine stimulation. Both D- and L-configurations of oligomeric lysine (upto 6–7 residues; mol. wt. 1000) supported comparable cellular expansion at 48 hr. We examined whether lysine acts independently as a cellular growth/division promoting agent or not. It probably acts in conjunction with the serum derived growth factors. This was evident from viable cellular expansion data at 48 hr in serum containing media versus serum-free one. Supplementation of the serum-free medium with insulin, transferin and selenite (ITS) in contrast (to QBSF alone), supported the characteristic 48 hr response *in vitro*. This evidently shows an indirect facilitating role(s) of the molecule at cell surface, possibly, through electrostatic binding of serum derived growth factor(s) just prior to or as a part of ligand receptor interaction (Figure 1) (Datta et al., 1997b, 2000). Least energy configuration model of 6 residue poly-L-lysine (molecular weight ~1000) showed characteristically close molecular orientation (twisting) in a plane of positively charged – NH_3^+ groups. TGF-β(2) (recombinant human homodimer molecule), a known agent promoting wound healing, also showed typical lysine residue concentrate (4 to 5 in number) at each end. It is obviously interesting to speculate an active role for this lysine concentrate in TGF-β and its receptor binding, in its biological expressions. This corroborates quite well with the experimental data of poly-lysine only upto molecular weight 1000 and not beyond, acting as cellular growth promoting agent *in vitro* (Datta et al., 1997b, 2000a).

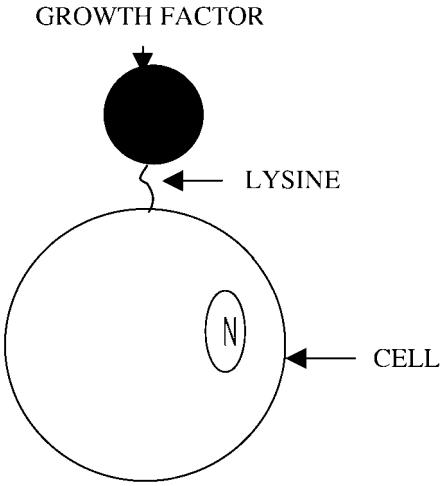

Figure 1. Mechanism of action of lysine.

Wound healing

In this world of cosmesis, a faster and scarless wound repair is much desired. A want is still felt for a molecule which can improve the rate and quality of healing without being prohibitively costly and having easy availability and high degree of cell/tissue compatibility.

A good amount of work has been done in the field of augmented wound healing using growth factors, which have known mitogenic potential. Epidermal growth factor (EGF) has been tried in improving wound strength and accelerating the rate of repair (Zhou et al., 1997). Delivery of platelet derived growth factor (PDGF) using cultured dermal fibroblasts transduced retrovirally with PDGF-B gene and adenoviral mediated gene transfer has been tried to overcome the ischaemic defect in wound healing (Breitbart et al., 1999; Liechty et al., 1999). Granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) also has been claimed to show promise (Jaschke et al., 1999; Grzybowski, 1999). Keratinocyte growth factor-2 (KGF-2) has been shown to accelerate wound healing with increase in wound strength and no obvious scarring (Junienez and Rampy, 1999). Basic fibroblast growth factor -β (bFGF) carried on a collagen matrix for inhibiting wound contracture has been tried out in diabetic and decubitus ulcers (Ono et al., 1999). TGF-β delivered through a collagen scaffold has also been tried in enhancing wound healing (Pandit, 1999). There is support for addition of protease inhibitors in conjunction with any treatment using growth factors (Trengove et al., 1999). Collagen and hyaluronan have been used by many as carriers for growth factors. Both per se have been used for tissue augmentation and wound healing (Ruszczak and Schwartz, 1999; Baichey et al., 1995; Jia et al., 1998). Platelet release treatment has also been tried in improving skin healing in diabetic rats through endogenous growth factor secretion (Moulin et al., 1998).

Accelerated wound healing has been seen in mice with a disruption of thrombospondin 2 gene (Kyriahides et al., 1999). Mice lacking Smad 3 have also shown accelerated healing (Ashcrott et al., 1999b).

Chitosan-heparin membrane is claimed to cause increased stabilization and concentration of growth factor in wound area. Immobilized heparin has also been shown to induce accelerated healing (Kratz et al., 1998). Water-soluble chitin has also been used as a wound-healing accelerator (Cho et al., 1999). It gave high tensile strength and arrangement of collagen fibres similar to normal skin. It has also been shown that positively charged dextran beads stimulate wound healing (Tawil et al., 1999). Thymosin beta 4 also has been shown to accelerate wound healing by increasing re-epithelialisation by stimulating keratinocyte migration, increasing collagen deposition and causing angiogenesis (Malinda et al., 1999).

A synthetic analogue of prostaglandin E_2 (PGE_2) has also been evaluated for its effect on wound healing. It was proposed it might be beneficial during early stages of inflammation rather than during later stages of remodelling (Talwar et al., 1996). Silver has also been shown to aid healing in sterile skin, although its mechanism of action is unknown (Lansdown et al., 1997). Topical estrogen has also been shown to accelerate cutaneous wound healing especially in aged humans associated with altered inflammatory response (Ashcroft et al., 1999a). Arnebin-1, a natural product isolated from *Arnebia mobilis*, has been shown to accelerate normal and hydrocortisone induced impaired wound healing (Sidhu et al., 1999a). Curcumin (diferuloyl methane), a neutral product from rhizomes of *Curcunia procera*, also showed enhanced wound healing (Sidhu et al., 1999b). The healing potential of *Calotropis procera* is also being evaluated (Rasik et al., 1999).

L-lysine HCl has shown remarkable improvement in both the rate and quality of wound healing. The molecule gives qualitatively better and much quicker wound healing with less scar and deformation in clean-cut model. Another remarkable feature of the

molecule is its ability to support healing process in really protracted wounds, e.g. in leprotic ulcer, long standing diabetic foot ulcer, etc.

On histopathology, lysine treated wounds showed a remarkable thickening of the dermo-epidermal layer, suggesting increased cell proliferation from the basal layer (Keratinocytes). From ^{14}C-glycine incorporation experiments, it was evident that in experimental wounds, nonspecific fibrous tissue formation and laying in and around the immediate vicinity of the wounds was less than the controls although both the experimental and the control wounds showed reasonable collagen laying in response to the wounds. Collagen synthesis in free skin of the same animals, away from the wound sites, was comparable in both the groups.

Topically lysine treated wound sites (chronic wound beds), showed a controlled degree of inflammation and angiogenesis process whereby, possibly, required extent of cellular and serum growth factors entry to the wound beds were ensured, thereby augmenting the healing process *in situ*.

It was postulated that lysine probably acts as a nonspecific binding molecule between the cell and it is a growth factor thereby promoting increased cell proliferation (Figure 1). This enhanced cell proliferation is probably similar to that observed in the scarless foetal repairs where, because of extremely high rate of cell proliferation, probably, the wounds normally get more filled up with cellular mass rather than by matrix material (Datta et al., 2000b, 2001; Datta, 2000; Datta and Kundu, 1999) with subsequent remodelling.

This contrasts sharply with the current trends in the development of wound healing products and processes: (i) where, mostly, one growth factor (Regranex from J&J) is delivered to the wound site or (ii) where one particular cell type (platelet) is delivered to the wound bed (e.g. from Cytomedix Inc.) or (iii) where the *in vitro* process of replacement material development is elaborate time consuming and costly (e.g. Apligraf from Organogenesis Inc.). The unique aspect of the wound healing property of the molecule (and its derivatives) lies in the fact that it does not interfere in the fundamental process of healing and helps the process peripherally by augmenting angiogenesis.

Angiogenesis

Therapeutic angiogenesis is being attempted in animal model by exogenously added vascular endothelial growth factor (VEGF) and other angiogenic growth factors. Therapeutic efficacy of simply injecting naked plasmid DNA or proteins into ischemic tissue to deliver secreted angiogenic factors holds promise (Marty and Risau, 1999). Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for VEGF-121 has been shown to induce focal angiogenesis sufficient to normalise blood flow to ischaemic myocardium (Lee et al., 2000). Transfection of human HGF gene into infarcted myocardium has been shown to induce a beneficial response to the decreased endogenous HGF, thereby causing angiogenesis (Aoki, 2000). A single intrapericardial injection of FGF-2 in a porcine model of chronic myocardial ischaemia has been shown to cause functionally significant angiogenesis without any adverse outcomes (Laham et al., 2000). PR39 and related compounds are claimed to be potent inducers of angiogenesis by selectively inhibiting inducible factor-1 alpha (Li et al., 2000).

Gene therapy to stimulate angiogenesis is supposed to be an effective way of bypassing occluded arteries (Isner, 1999). Direct *in vivo* gene transfer using a replication deficient adenovirus vector has been carried out in an animal model. It has shown sustained and localised expression of a potent angiogenic mediator (Magovern, 1996). Autologous transplantation of bone marrow cells improved damaged heart function by inducing angiogenesis (Tomita et al., 1999).

Local injection of angiogenic growth factors into the ischemic site have been attempted by various routes like catheter based transendocardial injection, intrapericardial delivery etc. (Kornowski et al., 2000). Intramyocardial injection of bFGF has been shown to salvage myocardium in the border zone (Aaregawa, 1999). Transmyocardial laser treatment has been shown to cause angina relief by destruction of myocardial peripheral nerve endings and improving perfusion through induction of angiogenesis (Tjomsland, 1999).

On histopathology, topical application of L-lysine HCl has been been shown to induce profound angiogenic response in cutaneous acute and chronic wounds. Moreover L-lysine HCl supports maximum cellular growth and expansion in serum containing media than in a serum-free media suggesting an action mediated through growth factors (Datta and Kundu, 1999; Datta et al., 2000a, b, 2001; Datta, 2000). Given that the molecule is basically a C-5 species with a chiral carbon (with an attached amino group and linear R-group of 4 carbon chains with terminal amino group), the amino acid should work as a non-

specific binding/attachment molecule between any cell and it is dedicated growth factor. Exactly this has been observed in all cell types – both in suspension and adherent cultures as well as *in vivo* repair wounds and inducing angiogenesis. Lysine mediated angiogenesis is postulated to be an end result of the molecule acting as a cell surface bridge binding the angiogenic factors to their receptors (Figure 1). It would be interesting to study details of angiogenic response in ischaemic myocardium where normally angiogenic factors are available at a higher concentration than adequately perfused myocardium (Laham et al., 1999, 2000). Given that the basic molecular mechanism of angiogenesis is universal and involves binding of the angiogenic factors to their receptors, the much augmented angiogenesis induced by the molecule may be the result of ligand-receptor binding process (Figure 1) (angiogenic factors and their receptors) getting more stable, widespread and predictable under the influence of this 'new molecular bridge' compared to the normal stochastic process.

Food additives

Food additives in a broad sense just means any substance added to food. It refers to any substance, the intended use of which results or may reasonably be expected to result directly or indirectly in its becoming a component or otherwise affecting the characteristics of any food. This definition includes any substance used in the production, processing, treatment, packaging, transportation or storage of food. If a substance is added to a food for a specific purpose in that food, it is referred to as a direct additive. For example, the low-calorie sweetener aspartame, which is used in beverages, puddings, yogurt, chewing gum and other foods, is considered a direct additive. Some additives are manufactured from natural sources such as soybeans and corn, which provide lecithin to maintain product consistency, or beets, which provide beet powder used as food colouring. Other useful additives are not found in nature and must be man-made. Artificial additives can be produced more economically, with greater purity and more consistent quality than some of their natural counterparts.

Prebiotic agents are food additives which mainly consists of oligosaccharides and dietary fibres, mainly inulin, and are potentially benefial in a number of ways: 1) potential protective effects against colorectal cancer and infectious bowel diseases by inhibiting putrefactive bacteria (*Clostridium perfringens*) and pathogen bacteria (*Escherichia coli*, *Salmonella*, *Listeria* and *Shigella*), respectively; 2) improvement of glucose and lipid metabolisms; 3) fibre-like properties by decreasing the renal nitrogen excretion; 4) improvement in the bioavailability of essential minerals; and 5) low carcinogenic factor (Grizard and Barthomeuf, 1999).

Colour additives like sulfiting agents are sometimes used to preserve the colour of foods such as dried fruits and vegetables, and to inhibit the growth of microorganisms in fermented foods such as wine. They are also sometimes used in baked goods, condiments, snack foods and other products.

Erythorbates are food ingredients that are used to inhibit the change of flavour and colour in food when exposed to air, such as when a cut apple is exposed to air. Produced from sugar, erythorbates are similar in chemical structure to vitamin C. Two forms of erythorbates, erythorbic acid and sodium erythorbate, are commonly used in hot dogs, luncheon meats, cured meats, fresh pork, poultry, frozen bananas, dehydrated apples and other foods (Food Additives, 1994, in cooperation with Food and Drug Administration).

Various kinds of food additives are supplemented due to some beneficial response either in certain diseased states or normally to increase growth and performance. For example: Aminoleban EN contains branched-chain amino acids and is known to be benefical for the protein-energy malnutrition in cirrhotic patients (Meng et al., 1999). Cardiohel (Inrich production) which is a biologically active food additive has been recommended in Ischemic heart disease because it allows to reduce the dose of hypotensive and coronarolytic drugs by 35% and cardiac glycosides by 25%, thus lowering the risk of relevant side effects (Ivashkin et al., 1999). It has been proposed to fortify certain grain-based products with Iron EDTA to increase the bioavailability of iron in human diets and thereby improve the iron status (Heimbach et al., 2000). Nutritive factors like vitamin A and retinoids, vitamin D and selen are used due to important anticarcinogenic properties they have. The phytochemicals promise a lot in anticarcinogenesis to prevent cancer. Spice extracts act antioxidatively by food supplementation, and turmeric extract has been shown to have the ability to prevent the deposition of triacylglycerols in the liver (Sreter, 1999). Food such as blueberries and spinach which are high in antioxidant capacity have been examined for their effects to prevent and/or reverse age-related declines in cerebellar noradrener-

gic receptor function (Bickford et al., 1999). Mixture of amino acids has been seen to act on the pituitary to enhance the adrenocorticotropic hormone (ACTH), lutenizing hormone (LH), and follicle-stimulating hormone (FSH) response to corticotropin-releasing hormone (CRH) + gonadotropic hormone-releasing hormone (GnRH) (di Luigi et al., 1999). Ingestion of carbohydrate/protein before and after exercise and dietary supplementation of various nutrients e.g. protein, glutamine, branched chain amino acid, creatine, leucine etc. have been purported to promote gains in fat free mass during resistance training. Creatine and calcium beta-hydroxy beta-methyl-buthylate (beta-HMB) supplementation during resistance training have been reported to increase fat-free mass in athletic and nonathletic populations (Kreider, 1999). Simplesse, a fat replacement (GRAS status confirmed by FDA in 1990) made from milk or egg white protein and olestra, which would partially replace fat have been evaluated.

Food additives have a great role to play in livestock production and animal feed industry. Added dietary pyridoxine has been shown to improve weanling pig growth performance (Woodworth et al., 2000). Oleamide has been reported to elevate milk oleic acid when fed to lactating Holstein cows (Jenkins, 2000). Cows fed diets supplemented with ruminally protected lysine and methionine showed increase in percentage of milk protein but no effect on production of milk, milk fat, and milk lactose. Infusion of isoleucine resulted in a higher milk lactose proportion and tended to produce more milk and milk lactose (Robinson et al., 1999). Alpha-ketoglutarate, precursor of glutamine, in combination with ornithine has been suggested to improve gut morphology and functions and counteract trauma-induced dysimmunity and exert anabolic/catabolic actions on protein metabolism. In combination they increase the synthesis of arginine, proline and polyamines that play key roles in metabolic adaptation to trauma (Cynober, 1999). Somatotropin has also been shown to improve body weight gain and feed efficiency (Tripp et al., 1998). Xylitol diet fed partially, also significantly prevented the reductions in body weight gain and food intake without affecting the early stage of inflammatory responses (Takahashi et al., 1999). Food additive ascorbic acid has been shown to have a protective effect against bacterial and viral diseases due to its effect on heterophil function (Andreasen and Frank, 1999). A previous study has shown that diet supplementation with thioproline, an intracellular sulfhydril antioxidant and free radical scavenger stimulates lymphocyte functions. It has been shown to enhance immune function whenever it is depressed (Correa, 1999). Arginine is said to hold a key position in the cellular functions and interactions that occur during inflammation and immune responses. L-arginine improves pain-free and total walking distance in case of claudication in peripheral arterial disease due to enhanced activity of endothelium derived nitric oxide (Maxwell et al., 2000). Dietary supplementation with L-arginine is said to modify reactivity of endothelium and smooth muscle by at least two mechanisms: one associated with activation of potassium channels and the other with receptor-coupled release of nitric oxide (Ray et al., 1999). Lysine in combination with arginine is said to be used by bodybuilders for the combination's alleged effect of stimulating the release of growth hormone. According to the European Federation of Animal Feed Additive Manufacturers (FEFANA), increased use of amino acids combined with reduced crude protein levels in feeds is estimated to have the potential to reduce nitrogen excretion by up to 20–25%. This is seen as one of the major opportunities for reducing nitrogen pollution. Published work by Brudevold and Southern (1994) showed, in a series of five experiments, that diets containing 12% crude protein but supplemented with crystalline lysine, threonine, glutamic acid, methionine, histidine, isoleucine, tryptophan and valine, were able to support similar performance in 10–20 kg pigs to conventional control diets containing between 19–22% crude protein.

Animals in which high rates of lean tissue growth are achieved through either genetic selection or the application of growth hormones and repartitioning agents, lysine requirements are substantially higher (Goodband, 1990). In a report by Easter (1994) it has been shown that the level of dietary protein in a standard maize soybean meal can be reduced by as much as four percentage units if the diet is adequately fortified with lysine, tryptophan and threonine.

Nutritional supplementation with lysine is also recommended in case of herpes simplex infection. The amino acid lysine, in the amount of 1–3 g per day, is effective in inhibiting the recurrence of herpes simplex infections in some individuals (Flodin, 1997).

Food preservatives

A long shelf life, antimicrobial properties, no adverse reactions, harmless metabolites are some of the qual-

ities desired of a food preservative agent. Though currently many are in use, each is beset with a drawback of its own. Hence the search for an ideal and may be natural preservative continues.

The use of food preservatives, such as benzoic acid, nitrites, and sulphites, as antimicrobials, and butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbic acid and tocopherols, as antioxidants, has probably changed food production patterns and eating habits more than has the use of any other class of food additive. These food preservative chemicals confer substantial benefits on man, not only by the preservation and increased palatability of food, but also by affording protection against the pathological effects of reactive oxygen species (ROS) which are associated with cancer, cardiovascular disease and aging. Nevertheless, although most preservatives are now considered to be without potential adverse effects there have been problems concerning the safety of some of these chemicals. Benzoic acid and sulphites probably cause allergies. The formation of nitrosamines from nitrites, which are carcinogenic, and the possible rodent carcinogenicity of BHA and BHT have cast a shadow over the safety of these agents (Parke and Lewis, 1992).

BHT and BHA antioxidants are capable of aggravating symptoms in certain patients with chronic urticaria (Goodman et al., 1990). The metabolites of BHT, 2,6-di-tert-butyl-p-benzoquinone (BHT-quinone), 2,6-di-tert-butyl-4-hydroperoxyl-4-methyl-2, 5-cyclohexadienone (BHT-OOH), and 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO) are believed to be carcinnogenic. BHT-OOH participates in oxidative DNA damage directly, whereas BHT-quinone causes DNA damage through H_2O_2 generation, which leads to internucleosomal DNA fragmentation (Oikawa et al., 1996).

Weak organic acid food preservatives are shown to exert a strong pro-oxidant action on aerobic yeast cells. In addition these acids are mutagenic toward the yeast mitochondrial genome, even at levels that are subinhibitory to growth. This has raised the concern that the large-scale consumption of these preservatives in the human diet may generate oxidative stress within the epithelia of the gastrointestinal tract (Piper, 1999).

Animal and *in vitro* studies have shown that vitamins C and E can effectively inhibit the formation of carcinogenic nitrosamines. Vitamin C supplementation has been reported to inhibit skin, nerve, and lung and kidney carcinogenesis. Vitamin E has been shown to inhibit skin, liver, oral, ear duct, and forestomach carcinogenesis. Several suggested mechanisms of action include modification of the metabolism of polycyclic hydrocarbons, reduction of mutagenic activity and reaction with genotoxic free radicals (Chen et al., 1988).

Parabens, methyl, ethyl, propyl, benzyl, and butyl, are the most common preservatives in use today. They are the alkyl esters of p-hydroxybenzoic acid and are used extensively because they are relatively nonirritating and nontoxic and offer good antimicrobial coverage (Pederse et al., 2000). However they have been shown to evoke an oestrogenic response *in vivo*, in sexually immature rainbow trout. Also, isolated cases of allergic contact dermatitis have also been reported. But considering their high efficacy and favourble preservative profile they are still the most preferred food preservatives (Mowad, 2000).

Nisin and pediocin PA-1 are examples of bacteriocins from lactic acid bacteria (LAB) that have found practical applications as food preservatives. Like other natural antimicrobial peptides, LAB bacteriocins act primarily at the cytoplasmic membranes of susceptible microorganisms. The dissipation of proton motive force is the common mechanism for the lethal activity of LAB bacteriocin. It has been proposed that nisin forms poration complexes in the membrane through a multistep process of binding, insertion, and pore formation (Montville and Chen, 1998). Combination of sodium lactate and nisin was particularly effective in reducing total bacterial counts in this food product. It also appears that this combination provides an increased protection against common pathogenic contaminants of fresh pork sausage, i.e. *Staph. aureus* and *Salmonella* species (Scannell et al., 1997).

Interactions of monolaurin, eugenol (phenolic compound) and sodium citrate (chelator) on the growth of common meat spoilage (*Lactobacillus curvatus, Lactobacillus sake, Leuconostoc mesenteroides, Brochothrix thermosphacta*) and pathogenic (*Escherichia coli* O157:H7 and *Listeria monocytogenes*) organisms were investigated. It was shown that the combinations of these chemicals were required to have any potent effect. The presence of sodium citrate was necessary to yield potent inhibition of *Lb. curvatus* and *Lb. sake* growth by the monolaurin and eugenol combinations (Blaszyk and Holley, 1997).

The effects of 5% potassium sorbate (PS) and 3% lactic acid (LA) applications on total mesophylic aerobic bacteria, total psychrotrophic aerobic bacteria, lactic acid bacteria, staphylococci and coliform bacteria, pH values, thiobarbituric acid (TBA) numbers,

and sensorial properties of vacuum-packed chicken leg and breast meats have been investigated during storage at 4±1 °C. In addition, residual sorbate was examined. A decrease in bacterial counts of chicken leg and breast meats was observed in the periods following the treatments of PS and LA; however, towards the end of the storage period, the effectiveness of PS was greater than that of LA. Quantities of sorbic acid found in the samples treated with PS were below the Acceptable Daily Intake established by the Food and Agriculture Organization/World Health Organization (Kolsarici and Candogan, 1995).

Welsh onion ethanol extracts were tested for their inhibitory activity against the growth and aflatoxin production of *Aspergillus flavus* and *A. parasiticus*. It was seen that the survival of spores of *A. flavus* and *A. parasiticus* depended on both the extract concentration and the exposure time of the spores to the Welsh onion extracts. The extracts have been shown to inhibit mycelial growth of two tested fungi cultured on yeast extract-sucrose broth. They also inhibited aflatoxin production in the culture at a concentration of 10 mg ml^{-1} or permitted only a small amount of aflatoxin production with extract concentration of 5 mg ml^{-1} after 2 weeks of incubation. Welsh onion ethanol extracts showed more pronounced inhibitory effects against the two tested aflatoxin-producing fungi than did the same added levels of the preservatives sorbate and propionate at pH values near 6.5. Thus they appear to potent biological preservative agents (Fan and Chen, 1999).

Lysine too has been found to play an interesting role as a preservative. Production of toxins A and B by *Clostridium difficile* is enhanced in a defined medium with biotin-limited conditions. Asparagine, glutamic acid and glutamine (10 mM) showed an effect on growth and toxin production similar to that of biotin. Lysine (10 mM) suppressed growth and inhibited toxin production. Addition of these toxin-inhibitory compounds within an incubation period of 2 days inhibited the enhanced toxin production, but later addition showed only slight inhibition of toxin production. Amino acids contained in the defined medium under the biotin-limited conditions were actively utilised in the presence of the three toxin-inhibitory amino acids, but in the presence of lysine, amino-acid utilisation was suppressed (Yamakawa et al., 1998). Homocitrate synthase is the first enzyme of the lysine biosynthetic pathway. It is feedback regulated by L-lysine. The effect of lysine inhibition was investigated in the strain *Penicillin chrysogenum*. Lysine decreases the biosynthesis of penicillin (determined by the incorporation of [^{14}C] valine into penicillin) by inhibiting and repressing homocitrate synthase, thereby depriving the cell of alpha-aminoadipic acid, a precursor of penicillin. Lysine feedback has been shown to inhibit *in vivo* the biosynthesis and excretion of homocitrate by a lysine auxotroph, L2, blocked in the lysine pathway after homocitrate. The molecular mechanism of lysine feedback regulation in *Penicillium chrysogenum* involves both inhibition of homocitrate synthase activity and repression of its synthesis. *In vitro* studies indicated that L-lysine feedback inhibits and represses homocitrate synthase both in low- and high-penicillin-producing strains (Luengo et al., 1980).

Thus it is seen that lysine has remarkable antimicrobial properties. Since lysine is an amino acid, the chances of any immunological response against it are negligible. These qualities of lysine can be employed to use it as an effective food peservative.

Adjuvants

Immunological adjuvants are agents that enhance specific immune responses to vaccines. The capacity to identify the nature and form of antigenic epitopes in proteins allows the specific design of vaccines to promote relevant protective immune responses. Such vaccines, although ideal in terms of specificity and purity, may not always achieve the desired levels of protection through failure to reach relevant cells of immune system due to simple dilution, elimination by host enzymes or lack of specific targeting. Many vaccines, currently under development and testing, are composed of synthetic, recombinant, or highly purified subunit antigens, which are often considered to be safer than whole-inactivated or live-attenuated vaccines. Concomitant with the above there has been development of a plethora of adjuvants aimed at enhancing responses to these ‘new’ immunogens (Mrsny, 1998). Parallely, there has been an almost equal rapid enhancement in understanding the complex nature of the immune learning response, particularly with respect to antigen processing, the nature and role of cytokines and the importance of dendritic cells and T-cell subsets in protection from infections.

Despite substancial progress in the areas of basic and applied immunology over the past couple of decades, and drastic modifications being introduced to the approach of vaccination, the area of vaccinology is probably facing a formidable and extremely

fundamental requirement of a biocompatible adjuvant. Although a number of them are available, none really fits the bill of human use, ideally.

Adjuvants can be grouped according to their physical characteristics and mode of action. They include particulate adjuvants, oil and emulsifier-based adjuvants, adjuvants providing controlled antigen delivery, adjuvants based on specific targeting of antigen, and gel-type adjuvants (Jennings et al., 1998). They may act non-specifically in promoting an immune response to an antigen through depot formation, or very specifically as in a 'delivery system' where an antigen is linked to a cellular protein, or targeted to a specific cell receptor. It has also become necessary that these differing approaches be combined, and an adjuvant/delivery system designed, to provide slow release of a targeted antigen (Jennings et al., 1998).

Adjuvants have diverse mechanisms of action and should be selected for use, based on the route of administration and the type of immune response (antibody, cell-mediated, or mucosal) desired for a particular vaccine. As mentioned, adjuvant mechanisms of action include: (i) increasing the biological or immunological half-life of vaccine antigens; (ii) improving antigen delivery and presentation; and (iii) inducing the production of immunomodulatory cytokines (2). Conventionally the efficiency of an adjuvant used to be measured by the capacity to induce enhanced antibody titres in serum and cell mediated immunity (CMI) to a given antigen. Recently, the capacity of an adjuvant is also measured by the quality as well as the magnitude of the induced immune response, where quality includes isotype and IgG subclass responses, T-helper cell responses characterized by the cytokine profiles and extent of cytotoxic T cell (CTL) induction. In the early phase of immunization some adjuvants influence the antigen administration and uptake by a so-called depot effect exemplified by aluminium hydroxide gel and oil adjuvants, which possibly is not as desired as alledged. A 'modern' depot is exerted by slow release formulations, continuously releasing the antigen over a period of time at a steady rate or by pulses at intervals, although being basically a 'single injection' vaccine. Extensive efforts are put in to formulate efficient delivery combinations targeting the antigens from the site of administration to draining lymph nodes or distant lymphatic tissue or to mucosal surfaces by parenteral or mucosal administrations. Nowadays, non-replicating carriers besides replicating vaccines are formulated to induce mucosal immune responses encompassing secretory IgA and CMI (Morein et al., 1996). Efforts to evoke immune responses on mucosal membranes distant from the site of administration have resulted mostly in little success (Morein et al., 1996). For a long time it was considered that CTL under the restriction of MHC Class I only could be evoked by replicating viruses or intracellular parasites (Morein et al., 1996). However, novel adjuvant delivery systems readily induce CTL by delivering the antigen to the APC resulting in intracellular transport to the cytosol for the MHC Class I presentation system, as well as to the endosomal pathway for the MHC Class II presentation (Morein et al., 1996).

Infant immunization is another particularly important area with multiple challenges for vaccine research and development. There is, together with a high susceptibility to infections, a lower efficacy of most vaccinations in newborns and young infants, compared to those administered later in life. Hence adjuvants play an important role to: (i) rapidly induce strong antibody responses of the appropriate isotypes; (ii) elicit sustained antibody responses extending beyond infancy; (iii) induce efficient Th1 and CTL responses in spite of the preferential Th2 polarization (in early life responses); (iv) escape from maternal antibody mediated inhibition of vaccine responses; (v) show acceptable reactogenicity in early life; and (vi) allow incorporation of several vaccine antigens into a single formulation so as to reduce the number of required injections (Kovarik and Siegrist, 1998).

Currently, aluminum salts and MF59 are the only vaccine adjuvants approved for human use. Of the novel compounds recently evaluated in human trials, immunostimulatory molecules such as lipopolysaccharide (LPS) derived monophosphoryl lipid (MPL) and saponin derivative QS21 appear most promising, although doubts have been raised as to their safety in humans (Singh and O'Hagan, 1999). Preclinical work with particulate adjuvants, such as the MF59 microemulsion and lipid-particle immune-stimulating complexes (Iscoms), suggest that these molecules are also potent elicitors of humoral and cellular immune responses (Singh and O'Hagan, 1999). In addition, preclinical data on CpG oligonucleotides appear to be encouraging, particularly with respect to their ability to selectively manipulate immune responses (Singh and O'Hagan, 1999).

MF59, an adjuvant approved for human use, typically elicits higher antibody titers than alum when used in combination with a variety of recombinant and natural subunit antigens. The mechanisms responsible

for the adjuvant action of MF59 however are not fully understood (Dupuis et al., 1999).

MPL has been shown to be a promising adjuvant due to the following advantages a) MPL retains the useful immunostimulating activities of the parent LPS molecule, but with greatly attenuated toxicity; b) produces diverse effects on the cellular elements of the immune system, including macrophage activation and T and B cell interaction, with concomitant cytokine and lymphokine release; c) proven adjuvant activity, in both the cellular and humoral effector arms of immunity; d) adjuvant activity when used alone, or in combination with other immunostimulants and delivery vehicles; and e) safe to humans (Ulrich and Myers, 1995).

Two novel oil adjuvant vaccines, Montanide ISA 25 and 206 are claimed to offer a number of advantages over $Al(OH)_3$, particularly in their ability to raise better immunity in pigs. Results indicate that vaccines, in presence of these adjuvants retain potency for a longer period and elicit good antibody responses in both pigs and cattle regardless of injection route without evidence of toxicity (Barnett et al., 1996).

It has been seen that formulation of a DNA vaccine encoding hepatitis B surface antigen with calcium- or aluminum phosphate adjuvants, increases antibody titers by 10 to 100-fold and decreases the immunogenic dose of DNA by 10-fold. Furthermore, boosting an HBs protein-primed response with the adjuvanted DNA vaccine resulted in a dramatic increase in the HBs-specific IgG2a response reflecting a shift towards a TH1 response. It has been suggested that the mechanism by which aluminum phosphate exerts its adjuvant effect is not through increased expression of HBsAg *in vivo* but by increasing the number and affinity of HBs peptide antigen-specific IFN-gamma and IL-2 secreting T cells (Wang et al., 2000).

Microfluidized squalene or squalane emulsions act as efficient adjuvants, eliciting both humoral and cellular immune responses. Squalene or squalane emulsions have been administered in human cancer vaccines, with mild side effects and evidence of efficacy, in terms of both immune responses and antitumor activity (Allison, 1999).

Water-soluble fullerene derivatives have been suggested as prospective adjuvants due to their immunostimulating effect (Masalova et al., 1999).

The role of Bacillus Colmette-Guerin (BCG) as an adjuvant in autologous tumor vaccines has been examined. BCG results in increased VPLN (vaccine primed lymph node) cell yield as well as enhanced type 1 (IFN-gamma release) immune responses of VPLN cells to autologous tumor without upregulating type 2 (IL-10 release) response (Li et al., 2000).

Experiments demonstrate that heat killed lysteria as an adjuvant for immunotherapy mediates immune deviation from a pathological Th2-dominated response toward a protective immune response in peripheral lymphoid tissues and in the lungs and may be clinically effective in the treatment of patients with established asthma and allergic diseases (Hansen et al., 2000).

The limited availability of efficient and non-toxic adjuvants, capable of promoting mucosal responses, presents with a problem in vaccinology. The potential usefulness of fibronectin-binding protein I (Sfbl) of *Streptococcus pyogenes* as immunological adjuvant has been assessed using ovalbumin (OVA) as a model antigen. Phenotypic analysis of proliferating cells show enrichment in $CD4^+$ T cells, producing a pattern of cytokines (IL-4, IL-5, IL-6 and IL-10) characteristic of Th2-type cells. In contrast to immunization with soluble OVA alone, OVA-Sfbl induced the generation of $CD8^+$ OVA-specific cytotoxic cells. These results demonstrate that Sfbl represents a promising mucosal adjuvant able to substantially improve cellular, humoral and mucosal responses when coupled to an antigen administered by intranasal route (Medina et al., 1998).

Norwegian outer membrane vesicle (OMV) vaccine against group B meningococcal disease has proved to be strongly immunogenic when administered intranasally in mice. All vaccinees developed marked increases in OMV-specific IgA antibodies in nasal secretions. It is thus possible that a nasal OMV vaccine may induce protection against invasive meningococcal disease, and also that it might be used as a vehicle for nasal vaccines against other diseases (Haneberg et al., 1998).

Whole killed meningococci (Nm) and pertussis bacteria (Bp) has been tested for mucosal immunogenicity and as mucosal adjuvants for an inactivated influenza virus vaccine given intranasally to unanaesthetized mice. With Bp or Nm admixed, serum IgG and IgA and salivary IgA responses to the influenza virus were substantially augmented ($P < 0.005$) (Berstad et al., 2000).

A study has been carried out to demonstrate that two mutants of *Escherichia coli* heat-labile toxin (LT), LTK63, which lacks ADP-ribosylating activity, and LTR72, which has partial enzyme activity, act as po-

tent mucosal adjuvants for the nasal delivery of an acellular pertussis (Pa) vaccine (Ryan et al., 1999).

Synthetically prepared N-terminal parts of the lipoprotein from Enterobacteria carrying three fatty acid residues or lipopeptide analogs containing one to four aminoacids bound to S-glycerylcysteine act as potent immunoadjuvants *in vivo* in combination with or covalently linked to antigens. Thus, bacterial cell wall components such as lipopolysaccharide, a variety of membrane proteins, murein, and lipoprotein can act as immunoadjuvants for bacterial vaccines, thus enhancing protection from bacterial infections. The immunoadjuvant properties of the lipopeptides are mediated by an enhancement of the humoral immune response (Schlecht et al., 1993).

Though vaccination has been the most cost-effective way of controlling infectious diseases, the logistics of delivering at least two to three doses of conventional vaccines for primary immunization to achieve protection are difficult and compliance is frequently inadequate, particularly in developing countries. In recent years biodegradable polymer microspheres have received much attention for the purposes of controlled release of antigens: (i) to reduce the number of doses needed for primary immunization to as few as a single dose and (ii) to better-target an antigen to microfold cells on mucosal surfaces after oral administration or to antigen-presenting cells after parenteral inoculations. A variety of vaccine antigens have been encapsulated in microspheres usually composed of poly (lactic/glycolic) acid (PLGA). Based on the size of the microspheres, molecular weight of polymer and ratio of lactic to glycolic acid in the polymer, the antigen may be targeted to various cells of the immune system or it may form a depot at the site of injection, allowing slow release of the antigen for extended periods. Additionally, an adjuvant may be incorporated inside microspheres together with the antigen, further enhancing or modulating the immune response to the desired type. The major problems in developing controlled-release vaccines include instability of vaccine antigens during micro-encapsulation, storage and subsequent hydration (Gupta et al., 1998).

Prolonged presence of mIFN gamma at the site of antigen presentation is crucial for the generation of systemic immune responses in the B16 melanoma model. Studies show that liposomal encapsulation of cytokines proves to be an attractive strategy for paracrine cytokine delivery in tumor vaccine development (Van Slooten et al., 2000).

Archaeosomes act as promising vaccine carriers capable of facilitating strong primary and memory responses, both in humoral and cell-mediated immunity sectors to entrapped antigens. In contrast, conventional liposomes induced little cell-mediated immunity, whereas alum stimulated only an IL-4 response. In contrast to alum and Freund's adjuvant, archaeosomes composed of Thermoplasma acidophilum lipids, evoked a dramatic memory antibody response to the encapsulated protein (at approximately 300 days) after only two initial immunizations (days 0 and 14). This correlated with increased antigen-specific cell cycling of $CD4^+$ T cells through increase in synthetic (S) and mitotic (G(2)/M) phase and decrease in resting (G(1)) phases of $CD4^+$ cell cycle (Krishnan et al., 2000).

One of the approaches of tumor vaccines is a mixture of irradiated tumor cells with cytokine containing liposomes. These vaccines are quite easy to prepare and, in contrast to vaccines consisting of cytokine-gene transfected-tumor cells, their composition (cell dosage, cytokine dosage) can be easily varied. Vaccination efficiency depended on (a) the immunogenicity of the tumor cells (b) vaccination frequency and (c) the dose of the cytokine encapsulated in the admixed liposome depots. Immunity to the tumors could be induced only within a narrow cytokine-dose range ('IL-2-dose window') (Krup et al., 1999).

Dendritic cells (DCs) have also been widely considered to be promising adjuvants for inducing immunity to cancer (Thurner et al., 1999). Studies using mature, monocyte-derived DCs to elicit resistance to malignant melanoma have been undertaken. In one of the studies, DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. This study proved the principle that DC 'vaccines' can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active $CD8^+$ CTL-tumor cell interaction *in situ* as well as escape by lack of tumor antigen expression (Thurner et al., 1999).

The co-delivery of inteferon (IFN)-gamma, interleukin (IL)-12, and IL-18 genes along with DNA vaccine constructs to engineer the immune response *in vivo* towards more T-helper Th1-type cellular responses, has been investigated. Co-immunization of IFN-gamma and IL-18 in macaques enhanced the level of antigen-specific antibody responses. Similarly, co-delivery of IL-12 and IL-18 also enhanced the level of antigen-specific Th proliferative responses (Kim et al., 1999).

The immunostimulatory sequences can also be identified within a pleiotropic cytokine like IL-1 and used in the rational design of novel vaccination strategies. The human interleukin-1beta (IL-1beta) domain in position 163-171, comprising the amino acids VQGEESNDK, has been synthesized as a nine-amino-acid-long peptide and used *in vivo* as a nontoxic HCl salt. The IL-1beta nonapeptide reproduces immunostimulatory and adjuvant effects of the whole mature IL-1beta, but does not possess any of the IL-1beta inflammatory, vasoactive, tumor-promoting, and systemically toxic effects, nor can it synergize with tumor necrosis factor alpha or other molecules in inducing toxicity and shock. Thus it acts as a promising adjuvant (Boraschi and Tagliabue, 1999).

IL-12 has also been investigated as a promising adjuvant. Its main effect is to drive Th-cell differentiation throughout a T-helper type-1 response, thus inducing interferon gamma (IFNgamma) and favoring IgG2a. These properties make IL-12 a candidate adjuvant for vaccination against cancer and infective diseases. But, experience of some toxicity in humans has hampered its further development into clinical applications, which, however, are still possible if restricted to local administration. Gene transfer has been proposed to be the preferred approach to obtain local release of cytokine (Rodolfo and Colombo, 1999).

Synthetic oligodeoxynucleotides containing CpG motifs [immunostimulatory sequences (ISS)] have been described as potent adjuvants of type 1 immune responses when co-administered with protein or peptide vaccines. ISS causes a rapid release of IL-12 and IFN-gamma in sera from treated mice. This data provide a first evidence for the ability of ISS to induce an anti-CHO (polysaccharide) type 1-like immune response and demonstrate that ISS have the potential to increase host antibody response against both the CHO and the protein component of a conjugated vaccine (von Hunolstein et al., 2000). Addition of CpG ODN (Oligodeoxynucleotides) to hepatitis B vaccine greatly increases the seroconversion rate and the titers of antibody against HBsAg (anti-HBs). This is the first demonstration of CpG DNA in a great ape and the results have important implications for the vaccination of humans against HBV and other diseases (Davis et al., 2000).

Studies have indicated that professional APCs in the periphery, such as dendritic cells and macrophages, play an important role in initiating DNA vaccine-specific immune responses. To engineer the immune response induced by DNA vaccines *in vivo* the modulatory effects of codelivering growth factor genes for the hematopoietic APCs along with DNA vaccines was investigated. Specifically, the effects on the antigen-specific immune responses following the codelivery of the gene expression cassettes for macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), and granulocyte macrophage-colony stimulating factor (GM-CSF) along with HIV-1 DNA immunogen constructs were studied. It was observed that coimmunization with GM-CSF increased the antibody response and resulted in a significant enhancement of lymphoproliferative response. Furthermore, among all coinjection combinations, M-CSF coinjections resulted in a high level of CTL enhancement. This enhancement of CTL responses observed from the coinjection with M-CSF was $CD8^+$ T cell dependent and was associated with the presence of $CD11c^+$ cells at the site of injection and with the antigen-specific induction of the beta-chemokine MIP-1beta, suggesting a role for this chemokine in CTL induction. These results suggest that hematopoietic growth factors should be further studied as potential adjuvants for *in vivo* modulators of immune responses (Kim, 2000).

In the study that is being carried in the authors' laboratory, monomeric lysine has been observed to augment the Ig responses irrespective of the type and size of the antigens. Heat killed Mycobacterium tuberculosis was observed to give a massive augmentation of Ig responses reproducibly in the presence of the monomeric aminoacid. BCG strains were also remarkably potentiated in their ability to induce humoral immunity. Few other antigens are being studied at the moment along with characterisation of the elicited humoral and cellular types of immunity including characterisation of the antibodies and cells (mostly $CD4^+$ and $CD8^+$) generated.

Antimicrobial peptides

Many peptides have antimicrobial activity. They encompass a wide variety of structural motifs. The majority of these peptides are cationic and amphipathic but there are also hydrophobic alpha-helical peptides, which possess antimicrobial activity. In addition, some beta-sheet peptides have antimicrobial activity and even antimicrobial alpha-helical peptides which have been modified to possess a beta-structure retain part of their antimicrobial activity. There are also antimicrobial peptides, which are rich in certain specific

amino acids (for example shepherin I and shepherin II, glycine- and histidine-rich peptides) (Park et al., 2000). There are antimicrobial peptides which are lipopeptides (for example Daptomycin) (Hodinka et al., 1987). In spite of the structural diversity, a common feature of the cationic antimicrobial peptides is that they all have an amphipathic structure which allows them to bind to the membrane interface (Epand and Vogel, 1999). Indeed, most antimicrobial peptides interact with membranes and may be cytotoxic as a result of disturbance of the bacterial inner or outer membranes (Epand and Vogel, 1999). Alternatively, a necessary but not sufficient property of these peptides may be their ability to pass through the membrane to reach a target inside the cell (Epand and Vogel, 1999). The interaction of these peptides with biological membranes is not just a function of the peptide but is also modulated by the lipid components of the membrane. It is not likely that this diverse group of peptides has a single mechanism of action, but interaction of the peptides with membranes is an important requirement for most, if not all, antimicrobial peptides (Epand and Vogel, 1999).

It has been seen that thiazole and oxazole containing amino acids like alanine, valine, proline, leucine and alanine and some peptides containing the 5-ring heterocyclic backbone modifications show moderate antibacterial activity *in vitro* against various Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, etc.) and Gram-negative (*Escherichia coli*, *Protens vulgaris*, etc.) bacteria, fungi (*Candida albicans*), and yeast (Stanchev et al., 1999).

The derivatives of cholic acid with basic aminoacids also show significant antimicrobial activity, especially marked when L-arginine is the condensed aminoacid (Bellini et al., 1979).

Macrocyclic peptides possess specific and potent antimicrobial activity that is salt dependent (Tam et al., 1999). It has been suggested that their initial interactions with the microbial surface may be electrostatic, an effect commonly found in defensin antimicrobial preptides. They have an end to end cyclic structure with a cystine-knot motif. It has been suggested that this might provide a useful template for design of novel peptide antimicrobials (Tam et al., 1999).

Nisin, an amphiphilic peptide, shows a strong antimicrobial activity against various Gram-positive bacteria. Its activity results from permeabilization of bacterial membranes, causing efflux of cytoplasmic compounds. It has been suggested that pore formation of nisin involves translocation of the C-terminal region of the molecule across the membrane (Van Kraaij et al., 1998).

A family of aminoacyl alkyl citrate compounds called viridiofungins, which are novel squalene synthase inhibitors, have broad spectrum fungicidal activity but lack antibacterial activity. Although the compounds inhibit squalene synthase, the first committed step in ergosterol biosynthesis, results presented show that inhibition of fungal growth is not related to inhibition of ergosterol synthesis (Onishi et al., 1997).

Novel pseudopeptides, corresponding to a membrane active depsipeptide, show more resistance to serum proteases than peptides and similar antimicrobial activities, without hemolytic activity. The pseudopeptides were found to be active against current drug resistant fungi and pathogenic fungi isolated from patients, and also have a strong synergism with current antifungal drugs against *Candida albicans*. The leakage assays suggest that the pseudopeptides act on the lipid membrane of pathogenic cells. Thus it is believed that they would have advantage over the peptide(s) as candidate(s) for novel antifungal(s) (Oh et al., 1999).

Peptoids, which differ from peptides in having a N-substituted rather than alpha-carbon-substituted glycine units, have been evaluated for antimicrobial activity. It has been seen *in vitro* that the peptoid CHIR29498 and some of its analogues were active in the range of 3 to 12 μm ml^{-1} against a panel of gram-positive and gram-negative bacteria which included isolates which were resistant to known antibiotics. Beta-Galactosidase and propidium iodide leakage assays indicate that the membrane is the most likely target of activity. Positional isomers of an active peptoid were also active, consistent with a mode of action, such as membrane disruption, that does not require a specific fit between the molecule and its target (Goodson et al., 1999).

Calprotectin, a protein in neutrophil cytoplasm and abscess fluids, appears to inhibit microbial growth through competition for zinc. It has been suggested that calprotectin's antimicrobial activity may be related to certain histidine-based zinc-binding sequences (Loomans et al., 1998).

A novel 26-residue proline-rich immune-inducible peptide, Metchnikowin, has been characterised from Drosophila, which exhibits both antibacterial (gram-positive) and antifungal activities (Levashina et al., 1995).

The antibacterial effect of o-carboranylalanine (o-Cba), a highly lipophilic analog of phenylalanine,

against some species is comparable to that of the widely used agricultural antibiotic, streptomycin. This carborane-containing amino acid is more toxic to Gram-positive bacteria, than to gram negative ones. Compared to the commercial fungicide, prochloraz, o-Cba is found to be weakly toxic against various fungi (Oros et al., 1999).

Two novel phenylalanine-rich antimicrobial peptides, Styelin A and Styelin B, purified from the hemocytes of Styela clava, are effective against a panel of gram negative and gram positive bacterial pathogens of humans, usually acting with minimal inhibitory concentrations of <1.5 μg ml^{-1} (<0.5 μM), even in the presence of 100 mM NaCl. The presence of antimicrobial peptides (Styelins) in tunicate hemocytes is evidence that such molecules are ancient mediators of host defense within the vertebrate lineage (Lee et al., 1997).

L-methionine has long been known to prevent reinfection with chronic urinary tract infection. The therapeutic result is essentially due to its influence on bacterial cytoadherence (Funfstuck et al., 1997).

Dermaseptins s1, s2, s3, s4, and s5, a family of cationic (lysine-rich), amphipathic antifungal peptides of 28–34 residues, share a similar spectrum of lytic activity against the filamentous fungi that are responsible for opportunistic lethal infections that follow the immunodeficiency syndrome or the use of immunosuppressive agents. They exhibit marked differences in their potencies to arrest the growth of gram-positive and gram-negative pathogenic bacteria and yeasts (Mor et al., 1994). The mechanisms of antimicrobial actions of magainin 2, buforin II and poly L-lysine against various *Escherichia coli* strains have been studied. Poly L-lysine inhibits BL21, AD 434 and GroE$^+$/DnaK$^+$ growth without lysing the cell. Magainin 2 has a pore-forming activity on BL 21 and AD 434 membrane but could not inhibit the GroE$^+$/DnaK$^+$ growth in a nutrient-rich medium. Buforin II, which kills BL21 and AD 434 without cell membrane damage, lysed GroE$^+$/DnaK$^+$ to death. Once they were introduced into the cell by electroporation, all three peptides were able to inhibit cell growth at concentrations of 10 times lower than their MICs (Liang and Kim, 1999). The antimicrobial activity of racemomycin compounds tended to be stronger with increase in the number of beta-lysine moieties in the molecule (Inamori et al., 1990).

Novel unnatural amino acids with more positively charged and bulky side chain group than lysine residue were synthesised. The incorporation of this amino acid increased the resistance of the peptide against serum protease more than three times without a decrease in the antimicrobial activity. It has been suggested that the amino acids synthesized in this study could be used not only as a novel building block for combinatorial libraries of antimicrobial peptides, but also for structure-activity relationship studies about antimicrobial peptides (Oh and Lee, 1999).

An amphiphilic, cationic peptide composed of eight leucines and six lysines was synthesized by solid phase peptide synthesis (SPPS) and tested against *E. coli* O157:H7 grown in TSB. The peptide was bactericidal and bacteriostatic at concentrations of 50 and 25 μg ml^{-1}, respectively. An inhibitory effect was also observed against stationary phase cells. Intracellular K^+ and ATP depletion were also observed. These results suggest that the peptide increased the cell membrane permeability but it did not lyse the cells (Appendini and Hotchkiss, 1999).

It has been reported that a new group of diastereomers of short-model peptides (12 amino acids long) composed of leucine and lysine with varying ratios, possess several properties that make them potentially better than native or *de novo*-designed all L-amino acid antimicrobial peptides. Preliminary studies have revealed that modulating the hydrophobicity and positive charges of these diastereomers is sufficient to confer antibacterial activity and cell selectivity (Hong et al., 1999).

Oxalysine, a novel anti-fungal antibiotic, isolated from Streptomyces roseoviridofuscus showed a good activity against Candida parapsilosis when compared to Amphotericin-B and 5-Fluorocytosine. It was found that oxalysine 0.4 mmol L^{-1} did not significantly inhibit their incorporations into protein and/or DNA, but strongly inhibited the incorporation of (^{14}C)-adenine into RNA (Zhang et al., 1993).

L-lysine works as a good anti viral agent for herpes simplex. Supplementation of l-lysine is one of the best options available for the treatment of herpes simplex virus infections, especially oral forms. L-lysine is also much cheaper than antiviral drugs such as Acyclovir. L-lysine supplementation works by tilting the balance between lysine and arginine heavily in favor of lysine. This ameliorates herpes outbreaks because the herpes virus depends on the presence of arginine for its replication. Lysine, in the amount of 1–3 g per day, is effective in inhibiting the recurrence of herpes simplex infections in some individuals (Flodin, 1997).

Cell fixatives

Most laboratories use formalin, glutaraldehyde, ethanol, paraformaldehyde and Bouin's liquid for tissue fixation. Coagulative fixatives are less popular. However, problems with formalin fixation comprise delay of fixation and variations in the duration of the fixation mainly (Ezaki et al., 2000). Quantification of DNA extracted by microdissection of tissue sections can be used for qualitative PCR analysis. Formalin fixation, before microdissection significantly diminishes the amount of extractable DNA and may lead to less reliable results, even of qualitative PCR analysis (Serth et al., 2000). Also a major artifact induced by formaldehyde fixation is the masking of tissue antigens due to cross-linking of protein amino acid residues (Ezaki et al., 2000). Bouin's fluid has been shown to affect PCR analysis. The performance of PCR has been shown to decrease for samples fixed in Bouin's liquid for longer than 6 hr or after 48 hr of incubation in a vacuum infiltration processor (in which Bouin's liquid-fixed and formalin-fixed samples are mixed (Camilleri-Bruet et al., 2000). Glutaraldehyde affects lipids adversely. Classical procedure i.e.glutaraldehyde fixation followed by epoxy resin embedding, results in the loss of 73–91% of the tissue lipids (Maneta-Peyret et al., 1999).

But there is a better side to glutaraldehyde as a cell fixative. When gelatin films were treated with glutaraldehyde (GA) solution at 60 °C, free aldehyde groups were introduced in the film. The bonding strength of GA-crosslinked gelatin films (GA gelatin films) with biological tissue was assessed using porcine skins. It was found that bonding strength increased with increasing aldehyde content in the film (Matsuda et al., 1999).

Ninety-five percent (95%) ethanol is the standard cytological fixative used in many laboratories. Commercially available ethanol is expensive and not freely available in some institutions. Methanol, a tissue dehydrant, is also known to be a cytological fixative. Methanol has been shown to be as effective as ethanol for fixation of smears and cheaper (Kumarasinghe et al., 1997).

To establish a quantitative method for analysis of gene expressions in small areas of tissue after paraffin embedding, preliminary validation experiments with RT-PCR and Western blotting were performed using methacarn-fixed rodent tissues and a cultured PC12 cell line. It was shown that in addition to its advantages for immunohistochemistry, methacarn-fixed paraffin-embedded tissue has benefits for analysis of both RNAs and proteins in the cells of histologically defined areas (Shibutani et al., 2000).

Adequate preservation of the cells and matrix of mineralising tissues remains difficult, as organic components and initial mineral deposits may be lost during conventional processing for electron microscopy. An attempt has been made to decrease the processing time by using microwave irradiation (MWI). Rat molar tooth germs were fixed using standard fixatives in a microwave oven. Under electron microscopy, differentiating ameloblasts and odontoblasts, plasma membranes, mitochondria, rough endoplasmic reticulum, the Golgi complex, together with all other cytoplasmic organelles showed excellent preservation, especially microtubules, microfilaments and coated vesicles. Crystal-like mineral deposits were conspicuously present in relation to dentine matrix vesicles and collagen fibrils as well as in enamel matrix. Thus this promises to be a fast and effective method when mineralised tissue is involved (Massa LF, Arana-Chavez et al., 2000). Even when applied in immunohistochemistry, MWI greatly shortens the fixation, processing, and immunolabeling times without compromising the quality of ultrastructural preservation and the specificity of labeling (Rangell and Keller, 2000).

Integral immunohistochemical analysis of immune responses in frozen sections requires that, in addition to constitutively expressed membrane CD markers, less stable determinants should be reliably visualized.

Fixation with pararosaniline, as compared to acetone, resulted in better morphology of all tissues. Out of a number of determinant-tissue combinations, staining sensitivity and intensity were markedly increased for selected determinant-tissue combinations, e.g., for IL-4 in human spleen and CD40 in human and mouse spleen. These data show that pararosaniline is a useful alternative to acetone, resulting in superior morphology and specific staining for selected determinant-tissue combinations (Schrijver et al., 2000).

Acetone fixation at –18 °C with subsequent embedding in methyl-/butylmethacrylate is a reliable method for the routine processing of bone marrow biopsies. This method allows good conventional histological visualization of morphological details, which is comparable with other fixation procedures. The essential advantage of this method is that a wide range of monoclonal antibodies and polyclonal antisera can be used for immunohistochemical investigations for

diagnostic and scientific purposes. The addition of 5% polyethylene glycol 400 to the acetone minimizes freeze-related artefacts (Hantschick and Stosiek, 1998).

Poly-D-lysine (PDL) and Poly-L-Lysine (PLL) are synthetic molecules which have been used to enhance cell attachment to plastic and glass surfaces (McKeehan, 1984). For many anchorage-dependent cells, the nature of the culture substrate has a major effect on cell growth and the requirement for serum proteins. Tissue culture plastic has a net negative surface charge, which is produced by plasma treatment of the polystyrene (LaRocca and Barker, 1996). Many researchers have shown that serum-free or reduced serum cultures can be dramatically improved by coating the culture surface with positively charged polymers, i.e., PDL and PLL (Yavin and Yavin, 1974; McKeehan and Ham, 1976). Poly-lysine surface treatment improves adhesive properties by altering the charge on the vessel surface from negative to positive. In addition to promoting cell adhesion, Poly-lysine also enhances the adsorption of serum or extracellular matrix proteins to the culture substrate (McKeehan, 1984).

While both PDL and PLL are being widely used, PDL may be preferred for some cell types and applications because PDL, unlike PLL, is not broken down by the proteases released by cells in culture (Banker and Goslin, 1991). As PDL and PLL are synthetic molecules, they do not stimulate biological activity in the cells cultured on them. In addition, they do not introduce impurities carried by natural polymers (Ham and McKeehan, 1979).

Hybridoma

With rising costs in medical treatment, prevention of diseases have become more important than the cure. Hence the trend is towards early diagnosis so that therapy is initiated at the earliest. The field of diagnostics has over the years developed and honed itself into one of the most important sectors of clinical medicine. These techniques rely heavily on the ability of monoclonal antibodies (MAbs) for their specifity and affinity for the antigens.

Since the advent of hybridoma technology in 1975 (Köhler and Milstein, 1975), MAbs have rapidly become one of the most important humanised animal cell products. They have rapidly made inroads into diagnosis, research, therapy, and purification processess (Chua et al., 1994). However, production of MAbs is cost intensive. This is mainly due to the expensive media requirements, low maximum cell densities, relatively low productivities and elaborate and high-end downstream processing. This problem is further compounded by the fact that hybridoma cell lines are very unstable during prolonged cell culture, frequently giving rise to non-producer cells (Chua et al., 1994).

There are two major approaches to obtain higher yield of MAbs in culture. High density culture/large scale culture of hybridomas constitute one approach and the other is the enhancement of individual cellular productivity (Sugahara et al., 1991).

Last couple of decades, optimisations have been directed mostly to reactor modifications (Martens et al., 1992; Lu et al., 1995), entrapment of cells (Lee and Palsson, 1993), media modifications and modulation of addition/feeding so that metabolite formation is limited and kept to the minimum (Chua et al., 1994; Bibila and Robinson, 1995; Bushell et al., 1994; Hiller et al., 1994; Jo et al., 1992, 1993; Kurokawa et al., 1994). Also, quite a few unique additives have been attempted and this has enabled the bioprocess engineers to scale up bioreactors and achieved greater productivity both through stand-alone and coupled approaches (Hiller et al., 1994; Murray et al., 1996; Sugahara et al., 1994).

In high-density culture there is a two-pronged approach to enhance antibody yield:

(1) Increasing the specific antibody secretion rate per cell in culture.

(2) To increase cell culture time, cell concentration and hybridoma longevity thereby obtaining a higher yield.

A model of cellular stoichiometry based on estimated cell composition, product profile, micronutrient and ATP demand etc has been used to design nutrient feeds. It has been shown that the use of nutrient feeds in the form of concentrated complete media eliminates the labour and time associated with the identification of the limiting nutrients and the optimisation of the feeding strategy (Bibila and Robinson, 1995). Supplementation with complete concentrated medium has also shown to increase culture longevity (Jo et al., 1992, 1993). The beneficial effects of concentrated medium is offset by the high cost of formulation and build up of high concentration of toxic by-products like NH_3. Another aspect of using concentrated medium is the enhancement in osmolarity of

suspending fluid which is harmful to the cells (Bibila et al., 1994a).

Physico-chemical stimulation of hybridma cells in culture is one of the varoius approaches of enhancing MAb production. Of these, chemical additives have been tried extensively (Xie and Wang, 1994a, b; Maeda, 1992; Sugahara et al., 1997, 1998; Voigt and Zintl, 1999) along with physical manipulations (Chua et al., 1994; Sanfeliu et al., 1996; Ker-hwa Ou and Patterson, 1997).

Production of MAbs has been hyperstimulated by high osmolarity in eRDF medium. It was shown that the medium eRDF, with or without serum, could stimulate MAb production better than the basal RPMI, DMEM media (Chua et al., 1994). It has been shown that in the presence of high concentrations of glucose and glutamine the antibody secretion rate decreases as compared to low concentration of glucose (0.2 g l^{-1}) and glutamine (0.1 g l^{-1}) where there was an increase of three to fourfold in production of MAbs in culture (Kurokawa et al., 1994). Dichloroacetate has been shown to enhance the growth phase by 20 hr thereby achieving a higher viable cell count leading to enhanced MAb yield (Murray et al., 1996).

Number of serum-free media have been developed and their effects on the growth and production of MAbs have been studied (Moore and Hood, 1993). Completely protein free media have also been developed (Fike et al., 1991). The physiological changes that take place during the adaptation of cells to low serum concentrations and serum-free media are of interest because downstream processing becomes much easier as the contaminating proteins are absent and the metabolite build-up is also relatively low. Additions of amino acids cysteine with methionine, trytophan and isoleucine with valine and vitamin B12 has been shown to result in significant increases in viable cell concentrations (Hiller et al., 1994).

Basic proteins and poly-basic amino acids have been used to increase the yield of IgM MAbs from human-human hybridomas. Addition of histones H1, H2A and H2B enhanced the Ig productivity by 3.2-, 2.6- and 2.8-fold, respectively. Poly-basic amino acids (poly L-lysine) have also been shown to enhance the production whereas poly L-arginine did not (Sugahara et al., 1994). Stimulation with step changes of lysine added media showed a marked increase in viable cell density and simultaneous increase in MAb titre. This was observed with the cell line AE9D6 and the cells could be kept viable for over 1400 hr in the fed-batch culture (Datta et al., 1997a, b, c, d, 1999, 2000, 2001).

Subsequent work based on lysine mediated cellular expansion in high-density culture of hybridoma cells has resulted in few marked improvements in the area of high-value-low-volume biochemical production (mostly MAbs) where partitioned co-product ion of more than one species of MAbs was possible with much simplication of down-stream processing (manuscript in preparation).

Adoptive immune therapy and immunomodulation

Adoptive immune therapy involves the passive transfer of antigen-primed T cells to initiate an immune response. The T cells are extracted from the host, 'taught' to recognise the foreign antigen and then transferred back into the host to initiate an effective immune response.

An important application of this recent and mostly experimental mode of therapy is against cancer. Tumour immunology consists of two essential concepts: immune surveillance, which specifies the host immune reactions against tumour cells, and tumour immune escape, which refers to the tumour-cell evasion process against the host immune system (Sheu et al., 1999). Cytotoxic T cells have an important role to play in the immune response against tumour cells (Toes et al., 1994). Adoptive T-cell therapy involves the passive transfer of antigen-reactive T cells to a tumour-bearing host in order to initiate tumour rejection. Both $CD4^+$ and $CD8^+$ cells are capable of initiating tumour rejection after adoptive transfer. Several different culture methods have been reported that permit *in vitro* expansion of immune T cells while retaining tumour specificity in this field (Li and Chang, 1999; Chang and Shu, 1996). Future directions in this field involve the selective isolation and expansion of subpopulations of T cells critical to initiating tumour rejection, and the use of molecular techniques to generate effector T cells (Markiewicz and Gajewski, 1999).

Adoptive transfer of polyclonal, tumour-specific, IFN -*gamma*-producing $CD4^+$ T cells [T helper type-1 (Th1) cells] have been used successfully against disseminated lymphomas. A single injection of 0.5×10^6 A20-specific Th1 cells has been shown to eradicate disseminated A20 lymphomas and provide lifelong protection without inducing autoimmune disease (Egeter et al., 2000). Cytotoxic T cell immunotherapy has also been tried against invasive cancers.

A 27-year-old woman with systemic chemoresistant and radioresistant metastatic disease secondary to a recurrence of human papillomavirus (HPV)-18 infected cervical adenocarcinoma of the uterine cervix received adoptive transfer of peripheral blood T cells stimulated with HPV18 E7-pulsed autologous dendritic cells (DC). The patient received two infusions of cytotoxic tumour specific T cells at two weeks intervals, and *in-vivo* distribution of the T cells was followed by 111-oxine labeling and serial gamma camera imaging. Persistent accumulation of radioactivity in the lungs, which harbored extensive metastatic disease, was detected upto 120 hr. after the infusion (Santin et al., 2000). *In vitro* CTL induction, however, is difficult in patients with advanced cancer. But once the cells are induced successfully, some favourable clinical effects are seen by the adoptive transfer of such cell populations (Soda et al., 1999).

Viral infections are also being combated by adoptive transfer of T cells. However, immunocytotherapy for persistent viral infections has proven successful in animal models but less effective in humans. Adoptive transfer of T cells has been shown to effectively purge virus from all tissues. But, maintenance of $CD8^+$ T cell effective functions after adoptive transfer is directly proportional to the amount of co-transfered, virus-specific $CD4^+$ T cells (Berger et al., 2000). Infectious bronchitis virus (IBV) infection and associated illness may be dramatically modified by passive transfer of immune T lymphocytes. As determined by respiratory illness and viral load, transfer of syngeneic immune T lymphocytes protected chicks from challenge infections (Seo et al., 2000). *In vitro* generated primary antigen specific Tc1 effective cells, producing high amounts of IFN-γ, or resting Tc1 memory cells, generated from these effectors, were protective aginst lethal pulmonary influenza infection in mice. Highly activated CD62Llow Tc1 effectors accumulated in the lung with rapid kinetics and most efficiently reduced the pulmonary viral titre early during infection (Cerwenka et al., 2000). Prophylactic administration of Ebstein Barr virus specific cytotoxic T lymphocytes (EBV-CTL) early after bone marrow transplantation (BMT) in humans appears to provide the most effective protection against the development of EBV-associated lymphoproliferative disease. Administration of EBV-CTLs before the onset of the EBV-DNA peak has been shown to stabilise virus titres within two to three logs above the normal levels. Administration of two to four infusions of 10^7 EBV-specific cytotoxic T lymphocytes CTLs/m^2 starting from the time of maximal virus load resulted in a 2- to 3-log decrease of virus titres (Gustafsson et al., 2000). Thus adoptive immune therapy appears to have an important say in the therapy against viral infections.

Another important application is against parasites. Experiments involving adoptive transfer of T cells have been successful in controlling malaria and toxoplasma infections in murine models. Adoptive transfer of $CD8^+$ T-cell splenocytes from Neospora caninum-infected mice was found to be protective against challenge with toxoplasma. The $CD8^+$ T-cells from Neospora-infected mice proliferated to both neospora and toxoplasma antigens *in vitro* and secreted substantial quantities of gamma IFN when pulsed with the parasite antigen (Kasper and Khan, 1998). In a murine model, it has been demonstrated that the adoptive transfer of intraepithelial lymphocytes (IEL) obtained from inbred mice at day 11 postinfection is able to protect against a virulent challenge by toxoplasma in syngenic recipients. A purified CD8alpha/beta$^+$ IEL population was isolated from infected mice at day 11 postinfection. These cells were able to protect naive mice by adoptive transfer against a lethal parasite challenge. RNA analysis by reverse-transcriptase PCR revealed that primed CD8alpha/beta$^+$ IEL produce significant message for IFN-gamma, an essential cytokine for host protection against toxoplasmosis (Buzoni-Gatel et al., 1997).

$CD4^+$T cells seems to play an important role in malaria infection. Cultured CD4+ T-cells that produce interferon gamma and IL-2, but not IL-4, in response to stimulation with the rodent parasite plasmodium berghei have been found to reduce but not eliminate parasites *in vivo* after adoptive transfer (Hirunpetcharat and Good, 1998). *In vitro* cultured $CD4^+$ T-cells, generated following immunisation with dead blood stage *Plasmodiium yoelii* parasites were found to mediate protection against homologous challenge infection in reconstituted nude mice. *P. yoelii*-specific T cell line cells produced IFN-gamma after *in vitro* stimulation with specific antigen, and were protective when adoptively transferred into athymic nude mice (Amante and Good, 1997).

Infectious diseases like leprosy have been combated by adoptively transferred T cells, although in a murine model. The model used was severe combined immunodeficiency (SCID) mice which lack both T and B cells. Cells from a known responder to mycobacterial antigens and from a non responder were activated by M. leprae cell wall antigens. The cells were harvested and co-injected with fresh M. leprae

into the right hind foot pads of SCID mice. After three months, there was no growth of M. leprae in the foot pads of mice co-injected with cells from the mycobacterial antigen responder, while growth was uninhibited in mice receiving cells from the non-responder (Converse et al., 1995). Dendritic cells (DC) are being tried to generate an effective immune response against mycobacterium tuberculosis infection. Conditionally immortalised dendritic cell line (tsDC) were infected with Mycobacterium tuberculosis. These activated DCs were shown to be capable of eliciting antigen-specific T cell responses and potent anti-mycobacterial protective immunity in a murine model of experimental tuberculosis infection (Tascon et al., 2000). To overcome the difficulty in generating specific CTLs, stable artificial antigen-presenting cells (AAPCs) are being developed that can be used to stimulate T cells of any patient of a given Human Leukocyte Antigen (HLA) type. Mouse fibroblasts are retrovirally transduced with a single HLA-peptide complex along with the human accessory molecules B7.1, ICAM-1, and LFA-3. These AAPCs consistently elicit strong stimulation and expansion of HLA-restricted CTLs (Latouche and Sadelain, 2000). Works are underway as for the role(s) of lysine and its analogues, acting as 'stimulating' co-stimulatory molecules in antigen presentation process (unpublished data). This seems interesting given the fact that the amino acid does act as a molecular bridge connecting cells and their growth factors (Datta and Kundu, 1999; Datta et al., 2000a, 2001). In a simultaneous study being carried out heat-killed MTb Ag showed varied types of immune reactions when administered with the amino acid given subcutaneously over a definite immunization regime. One of the main considerations of this immunomodulation capacity – both humoral and cell mediated – of the amino acid was whether it was injected separately or premixed with antigen (unpublished data).

Thus the important elements of adoptive immune therapy and immunomodulation are generation of stable antigen specific cells and their expansion. Lysine has been postulated to act as a non-specific bridging molecule. It holds a definite prospect in bridging the presented antigen to the T cell and thus ensure efficient priming of the T-cell against the antigen. Also, once antigen specific T-cells are created, the amino acid can possibly be used to bring about rapid cell proliferation expansion *in-vitro*. Thus a large bank of primed cells seems to be a distinct possibility using this molecule.

Tissue engineering

Tissue engineering is an emerging field focused on the creation of living tissues and organs for use in tissue repair and transplantation. Tissue engineering involves the fabrication of new functional living tissue using living cells which are seeded onto biocompatible scaffolds which can be natural, man-made or composite of both and grown under physiological conditions to produce human biointeractive implants. Tissue engineering is still in it's embryonic stage with a reasonable degree of challenges ahead. The scientific challenge here lies in understanding the cells themselves, including their behaviour in an altered environment, their mass transfer requirements and their interaction with the immediate milieu and the fabrication of compatible materials to provide scaffolding and templates. Large cell banks composed of universal cells that would be immunologically transparent to any individual are required. A viable alternative is to have autologous cells. Development of immunologically inert cells requires manipulation of these cells which still poses a great challenge. Advances in genetic manipulations might be required. These cells could be either differentiated ones or stem cell reservoirs which could then be signalled to differentiate into different lineages for structural application. These then need to be expanded to appropriate cell number before transfer to templates where further remodelling is expected to take place. A proper substrate for cell survival and differentiation is required. One of the strategies here is to use biocompatible implants composed of extracellular matrix molecules seeded with autologous cells or heterologous cells in conjunction with immunosuppressing drugs. Addition of growth and differentiation factors to these matrices as well as agonists and antagonists that favour cell matrix interactions can potentially increase the rate of successful tissue replacement. Collagen, either alone or in combination with other materials, is an important natural biomaterial that is used in a variety of tissue-engineering applications. The adhesiveness of collagen may be spatially controlled to allow controlled localization or redistribution of cells (Myles et al., 2000). Among one of the many appraoches involving different tissues, a biocompatible heterogeneous hydrogel of poly [N-(2-hydroxypropyl) methacrylamide] (PHPMA) matrix has been studied for it's neuroinductive and neuroconductive properties. It has the potential to repair tissue defects in the central nervous system by replacing lost tissue and by promoting the formation of a histotypic tissue mat-

rix that facilitates and supports regenerative axonal growth (Woerly et al., 1999).

Recently, tissue-engineering strategies have included cell and gene therapy. 'Cell therapy', use of cells to deliver active factors, is an emerging technique in the treatment of neurodegenerative disease. The method entails encapsulation into a hollow fiber device of discrete numbers of cell-containing microcarriers. Proliferation control is attained by embedding cell-containing microcarriers in nonmitogenic hydrogels. In one of the approaches, ability to control dose released over a several-fold range was demonstrated with encapsulated PC-12 cells delivering neurotransmitters and C2C12 mouse myoblast cells delivering neurotrophic factors (CNTF) (Li et al., 1999).

Tissue-engineering advances will be interdependent with advances in gene therapy techniques to restore function at a cellular level. The combination of tissue-engineering strategies with gene therapy approaches might allow transfection of diseased tissues with designated cDNA to eliminate inherent or acquired defects. Identification of the growth factors capable of directing tissue development and of the technique to be used for their delivery would aid in the engineering of human tissue (Amiel et al., 2000).

Tissue engineering strategies are being applied to replace various tissues and organs in various systems of the body. Tissue engineering of musculoskeletal tissues is a rapidly advancing field. In bone, technology has centred on bone graft substitute materials and the development of biodegradable scaffolds (Boyan et al., 1999). A composite matrix, containing esterified hyaluronic acid and gelatine, has been shown to facilitate the osteochondral differentiation of culture-expanded, bone marrow-derived mesenchymal progenitor cells. Thus, this composite matrix is useful for *in vitro* tissue engineering for repair of chondral and osseous defects (Angele et al., 1999). Important ongoing research is being aimed at tissue-engineering cartilage for surgical repair of tracheal defects (Van Veenendaal et al., 2000). Myoblast transplantation to date has been limited to the cellular level of replacement. It has been suggested that myoblast-polyglycolic acid constructs may be useful in defining the application of tissue engineering for future skeletal muscle transplantation. A study was conducted to tissue engineer three-dimensional vascularized skeletal muscle using isolated myoblasts attached to synthetic biodegradable polymer for tissue replacement in the enhancement of muscle regeneration. In this study, organisation of neomuscle strands surrounded by vascularized tissue composed of degrading polymer and fusing myoblasts demonstrated the ability of myoblast constructs to survive, reorganise and regenerate tissue-like structures (Saxena et al., 1999). An attempt has been made to culture ligament tissues *in vitro* by seeding human anterior cruciate ligament and medial collateral ligament cells onto synthetic biodegradable polymer fiber scaffolds. In the present study, mechanical stimulus and transforming growth factor enhanced proliferation whereas inflammatory agents had a negative effect (Lin et al., 1999).

Near term products, including injectable human matrix for contour defects and tissue-engineered cartilage, are proving to be important tools for plastic and reconstructive surgery (Naughton and Mansbridge, 1999). The development of tissue-engineered fat equivalents for reconstructive and augmentation purposes will be most welcome by nearly every surgical discipline and prove to be especially useful for plastic surgeons (Katz et al., 1999).

Improvement of cell culture conditions and functional cell expansion strategies in hepatic tissue engineering may permit cell/tissue banking and the generation of liver tissue equivalents for transplantation. Stimulatory effects of pancreatic islets on hepatocytes in co-culture for continuous hepatotrophic stimulation has been investigated in this context (Kauffman et al., 1999).

Bladder regeneration is another important area where extensive tissue engineering studies are carried out. Currently, two techniques for the induction of bladder regeneration are being researched. The first, *in vivo* technique, involves the use of a biodegradable scaffold that the host bladder can use to remodel and regenerate. This technique takes advantage of the cell's natural ability to heal or regenerate itself back to a normal state. The second technology, the *in vitro* technique, involves establishment of primary cell cultures from the host's native bladder. These cells are seeded on a biodegradable scaffold to create a composite graft that is then transplanted back into the host for continuation of the regeneration process (Kropp and Cheng, 2000). Tissue engineering for neointestine is also being studied. It has been shown that anastromosis between tissue-engineered intestine and native small bowel has a moderately high patency rate and a positive effect on maintenance of the size of the neointestine and development of the neomucosa (Kaihara et al., 1999). An attempt has been made to tissue engineer branched or bifurcated hybrid vascular prosthesis using bovine smooth muscle cells and type

I collagen with minimal reinforcement by a knitted fabric mesh made of segmented polyester. A branched hybrid graft with mesh reinforcement is expected to be applicable to arterial replacement in a branching region (Kobashi and Matsuda, 1999). A new type of a biodegradable nerve graft conduit material, the amnion tube, has been developed. The amnion tube is a potential ideal nerve conduit material secondary to its unique characteristics: it contains important neurotropic factors, is biodegradable, provokes a very weak immune response, is semiflexible, is readily available, and is easily manufactured into different sizes and diameters (Mohammad et al., 2000). Tissue engineering for bioprosthetic and mechanical valves and valved conduits poses quite a challenge because of its inability to grow, repair or remodel. An attempt to evaluate the feasibility of creating 3-leaflet, valved, pulmonary conduits from autologous ovine vascular cells and biodegradable polymers with tissue-engineering techniques has been tried (Stock et al., 2000).

Surface wound healing is another important area studied in the context of tissue engineering. Tissue-engineered skin implants have shown efficacy in a variety of wound care applications. Research efforts to modify cultured autologous skin transplants for large full-thickness burn wounds and in chronic ulcers are shifting from multilayered differentiated grafts ('sheet' grafts) toward smaller units of basal undifferentiated single cell suspensions in a transport medium and subconfluently covered static carriers (Voigt et al., 1999). A novel living skin replacement (LSR) biotherapy concept, combining the elements of cell therapy along with those of tissue engineering took advantage of the biodegradable microspheres, onto which donor skin epidermal and dermal cells could be attached and expanded *in vitro* for subsequent direct application down to the deepest recesses of the wound bed. This novel approach presents a number of advantages over existing therapies including facilitated cell manipulations, ease of storage and transportation, rapid clinical intervention due to the elimination of any surgical suturing or stapling, and a more natural three-dimensional tissue remodelling and anatomical compliance (LaFrance and Armstrong, 1999).

It is evident that the basic principle in tissue engineering is seeding appropriate number of cells after proper expansion on an appropriate substrate. This requires good cell proliferation to provide the appropriate cell number before seeding onto substrate and good cell viability. It has been observed that L-lysine HCl supports good cell proliferation both *in-vivo* and *in-vitro*, both in anchorage dependent and independent cells and it would be interesting to study any positive effects it might have in rapid expansion of autologous cells for development of bioengineered soft tissue prostesis. Autologous fibroblast layered bioprosthetic heart valve (aortic) development is in progress in the authors laboratory involving a new approach of fibre renewal in situ.

Gene therapy and DNA vaccination

Gene therapy has attracted much interest since the first submissions of phase I clinical trials in the early 1990s, for the treatment of inherited genetic diseases. Preliminary results were very encouraging and prompted many investigators to submit protocols for phase I and phase II clinical trials for the treatment of inherited genetic diseases and cancer. Also in 1990, the first gene therapy clinical trial for the treatment of patients with melanoma (Rosenberg et al., 1990) was conducted. The results of this study indicated that retroviral-mediated gene transfer in patients was safe. This finding prompted the submission of many other protocols for gene therapy clinical trials to treat patients affected by cancer, primarily in the area of melanoma (Osanto et al., 1993; Mahvi et al., 1997), followed by ovarian carcinoma (Deshane et al., 1997), sarcoma (Mahvi et al., 1997), brain tumor (Kun et al., 1995), and lung cancer (Nguyen et al., 1996). There is also a strong interest in beginning gene therapy clinical trials for the treatment of patients with AIDS, cardiopathies, and neurologic diseases. Indeed, gene transfer technology has already been applied in the phase I and phase II trials for the AIDS vaccine programs, which have recently begun in the U.S.A. (Haynes, 1996; Weber, 1996). As already anticipated, the spectrum ranges from the treatment of inherited or acquired genetic disorders to cancer, AIDS, cardiopathies, and neurologic diseases. This is strongly encouraging to the pursuit of gene therapy programs in medicine (stem cell).

There are a wide variety of vectors used to deliver DNA or oligonucleotides into mammalian cells, either *in vitro* or *in vivo*. The most common vector systems are based on retroviruses (Cournoyer and Caskey, 1993; Gilboa, 1990; Kohn et al., 1989; Miller, 1990; Temin, 1986), adeno-associated virus (AAV) (Berns et al., 1975; Cheung et al., 1980; Podsakoff et al., 1994), adenovirus (Karlsson et al., 1985; Yamada et al., 1985), herpes simplex virus (HSV) (Glorioso et

al., 1995), cationic liposomes (Thierry et al., 1992; Benne et al., 1992; Ropert et al., 1993). The stage of development of vectors and their variety are still not sufficient to be efficiently applied in therapy.

Among the other popular techniques for gene delivery, particle-mediated bombardment with a device called the gene gun has become an important developmental tool. This instrument has been used in numerous examples of wound gene therapy with growth factors or their receptors in the last decade. Among the advantages of particle-mediated bombardment are ease and speed of preparation of the delivery vehicle, the stability of the DNA preparation, the absence of (viral) antigens, the ability to target the projectiles to different tissue depths and areas, and the rapid shedding of both particles and DNA if they are targeted to the epidermis. Clinical application of the technology remains limited by the relatively low efficiency of the method, the potential tissue damage created by impact of the particles, and the coverage area (Kun et al., 1995). The field of gene therapy is at present actively involved in the challenging task of improving the design of vector systems for *in vivo* applications.

DNA vaccines have been used to stimulate protective immunity against many infectious pathogens, malignancies, and autoimmune disorders in animal models. Since their inception in 1950, there have been many research works done on the ability of DNA vaccines to induce strong immune responses against proteins from infectious agents such as malaria (Hoffman et al., 1997; Kalinna, 1997; Wang et al., 1998; Strugnell et al., 1997; Kaufmann, 1995; Lowrie et al., 1997), rabies virus (Xiang et al., 1994), hepatitis B virus (HBV) (Davis et al., 1994; Tacket et al., 1999), HSV (Kriesel et al., 1996), Ebola virus (Xu et al., 1998), and HIV (Boyer et al., 1997, 1999; Wang et al., 1993a, b). The strategy of most of these investigations is relatively simple: A DNA plasmid encoding a desired protein is injected into the muscle or skin of an animal, where it thereupon enters host cells and directs the synthesis of its polypeptide antigen. Once the plasmid-antigen is processed and presented by transfected host cells, a cellular and humoral immune response against the antigen is provoked. Genetic immunization exhibits many advantages over traditional vaccines that use live-attenuated or killed pathogen, proteins, or synthetic peptides: (a) Immunogenicity: can induce both humoral and cellular immune responses at low effective dosages (micrograms) in animal models. (b) Safety: Unability to revert into virulence unlike live vaccines and efficacy does not require the use of toxic treatments unlike some killed vaccines. (c) Engineering: Plasmid vectors are simple to manipulate and can be tested rapidly. Also, combination approaches can be easily adapted. (d) Manufacture: Conceptually low cost and reproducible large-scale production and isolation. Also can be produced at high frequency in bacteria and easily isolated. (e) Stability: More temperature-stable than conventional vaccines and has a long shelf-life. (f) Mobility: Ease of storage and transport (Shedlock and Weiner, 2000).

Another facet of DNA vaccine technology focuses on immune-related diseases, such as autoimmunity and cancer (Chen et al., 1999). By manipulating the balance of T helper (Th) 1 and 2 lymphocytes using DNA plasmid immunization, many of the pathogenic qualities of autoimmune disease may be potentially addressed (Ramshaw et al., 1997). DNA immunization has also proven as an effective candidate in the fight against certain cancers. The growth of human tumor cells that produce and secrete a target protein has been retarded or inhibited by a DNA vaccine construct encoding a subunit of that target protein (Geissler et al., 1997).

The most popular method of administering DNA vaccines has been parenterally, which includes needle injection into muscle or skin and gas-powered, DNA-covered particle bombardment using a ‘gene-gun’ (g.g.) (Shedlock and Weiner, 2000). Noninvasive methods of plasmid delivery involve the topical application of plasmid to the skin or mucosae. The induction of antigen-specific immune responses has been shown following the application of a plasmid solution to various mucosal surfaces including intranasal (Klavinskis et al., 1999; Hamajima et al., 1998), oral (Etchart et al., 1997), and intravaginal (Bagarazzi et al., 1998; Wang et al., 1997). Forms of delivery targeting the skin, including i.d. injection, g.g. bombardment, and topical application, have been shown to elicit a humoral response primarily, characterized by a rapid progression to a Th2-type response, associated with the production of an IgA and IgG1 antibody isotype (Fan et al., 1999; Boyle et al., 1997). Conversely, injection into muscle results in the induction of a strong cellular-mediated response, or Th1 type, that primes antigen-specific cytolytic T lymphocytes (CTLs) and is associated with the production of IgG2a antibody (Sin et al., 1999). DNA vaccines elicit strong and long-lasting humoral and cell-mediated immune responses in many animal models. At the cellular level, the efficacy of DNA vaccination de-

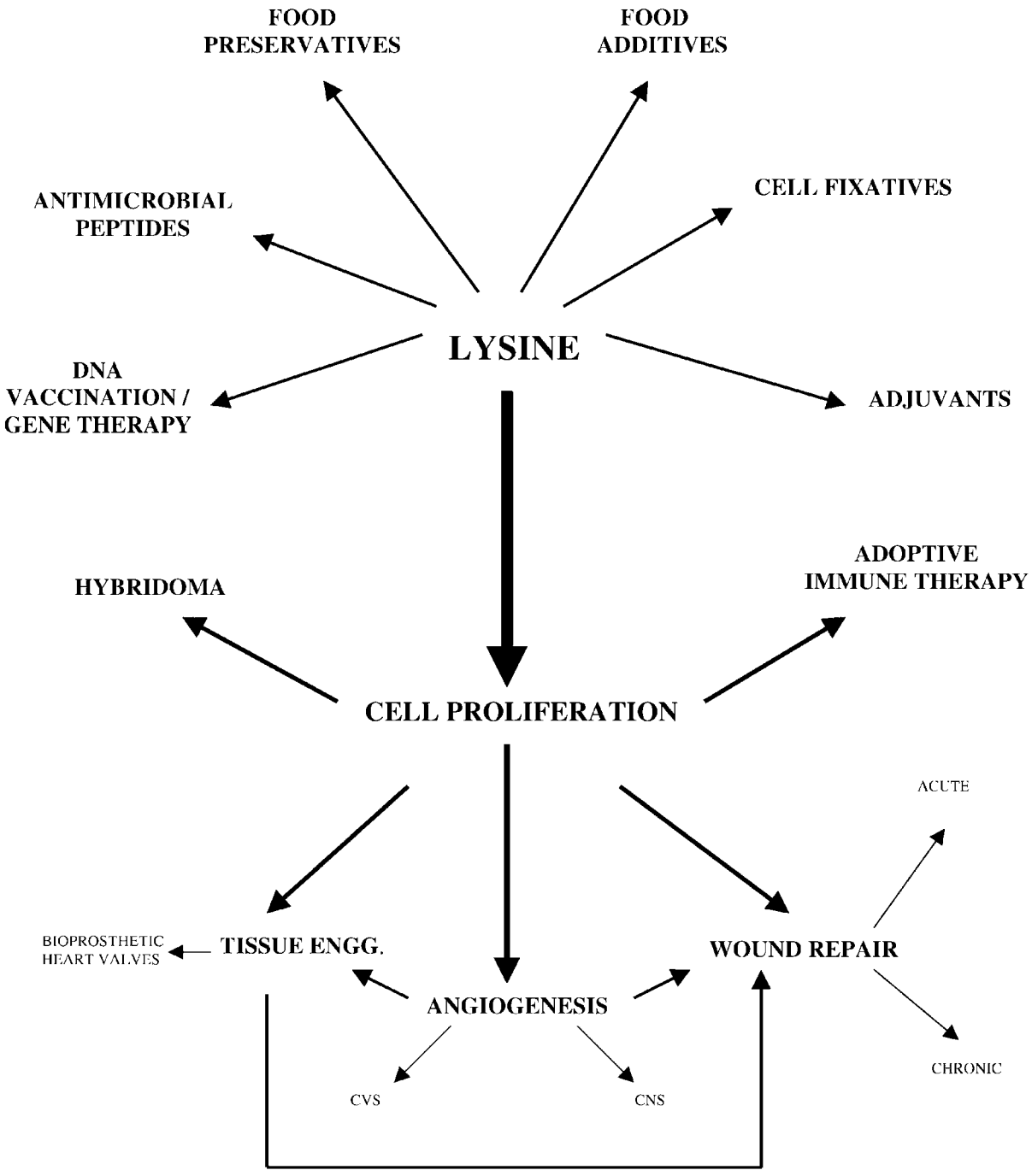

Figure 2. Schematic diagram depicting the biochemical roles of lysime.

pends on the interaction between their polypeptide products and the two major groups of cells that mediate immunity: lymphocytes and APCs. The codelivery of plasmids encoding costimulatory molecules is a method theorized to improve the antigen-presenting capabilities of transfected host cells. Another strategy currently under development is the coadministration of cell-surface molecules that induce cellular apoptosis. Theoretically, this technique targets vaccine antigen to the cross-priming pathway by delivering antigen associated with apoptotic cells to DCs and thereby guiding immune responses toward a Th1 phenotype (Chattergoon et al., 2000). Recently, expression of fusion proteins from a DNA vaccine is an attractive means of modulating an antigen-specific immune response without the use of potentially toxic chemical adjuvants (Shedlock and Weiner, 2000). Combination vaccination strategies are also being targeted nowadays. Many heterologous prime-boost strategies use DNA vaccines, recombinant virus, and protein in different assortments, which 'prime' the immune system to the vaccine antigen first, followed by a subsequent 'boost' immunization, which enhances the preliminary response. Strategies gaining the most attention are DNA vaccine-priming followed by protein or recombinant virus-boosting (Sin et al., 1999). Currently, many promising techniques of immune enhancement are being developed that modulate the intensity and direction of responses, such as the use of genetic adjuvants and DNA vectors of greater immunostimulation (Shedlock and Weiner, 2000).

Due to the hazards associated with viral vectors nonviral DNA complexes show promise as alternative and attractive gene delivery vectors for treating genetic diseases. Nonviral DNA complexes are typically formed by combining DNA with various condensing/complexing agents such as lipids, polyelectrolytes, polymers, polypeptides, and surfactants in solution. DNA/poly-L-lysine polyplex formation kinetics are probed by time-resolved multiangle laser light scattering (TR-MALLS), which yields the time evolution of the supramolecular complex mass and geometric size. Primary polyplexes whose geometric size is smaller than individual DNA molecules in solution are formed very rapidly upon mixing DNA and poly-L-lysine. Over time, these primary polyplexes aggregate into larger structures whose ultimate size is determined primarily by the relative concentrations of DNA and poly-L-lysine (Lai and Van Zanten, 2001). Polylysine (pLy) has been used as a DNA carrier in nonviral gene delivery systems because it forms complexes with plasmid DNA via charge interaction, and condenses it into a compact structure. It has been recently shown that cross-linking nuclear localization sequences (NLSs) to pLy can enhance transfection by conferring specific recognition by the cellular nuclear import 'receptor', the NLS-binding importin alpha/beta heterodimer. A clear correlation has been indicated between the pLy-DNA structure, importin alpha/beta recognition, and gene transfer efficiency, thus underlining the importance of using pLy-DNA at the optimal Ly/Nucleotide ratio (Chan et al., 2000). Poly-L-lysine has been shown to be a major contributor for gene transfer to hematopoietic progenitor cells, mediating the initial vector-cell binding. Human progenitor cell lines are poorly transduceable with recombinant adenovirus vectors. This new poly-L-lysine-modified, adenovirus-based vector could overcome virus tropism restrictions and consistently achieve very high transduction efficiency (>90%) in cells otherwise refractory to adenovirus gene transfer. Polylysine-based adenovirus vectors may have promise for situations in which high-efficiency gene transfer with transient high level transgene expression in hematopoietic cells is needed, such as leukemia vaccine protocols or for purging strategies in leukemia cell contaminated stem cell preparations (Schwarzenberger et al., 2001).

Modification of poly-L-lysine and poly-L-ornithine by the covalent attachment of palmitoyl and methoxy-poly (ethylene glycol) (mPEG) residues produces a new class of amphiphilic polymers-PLP and POP, respectively. These amphiphilic amino acid based polymers have been found to assemble into polymeric vesicles in the presence of cholesterol. *In vitro* biological testing revealed that POP and PLP vesicle-DNA complexes are about one to 2 orders of magnitude less cytotoxic than the parent polymer-DNA complexes although more haemolytic than the parent polymer-DNA complexes. The polymeric vesicles condense DNA at a polymer: DNA weight ratio of 5:1 or greater and the polymeric vesicle-DNA complexes improved gene transfer to human tumor cell lines in comparison to the parent homopolymers despite the absence of receptor specific ligands and lysosomotropic agents such as chloroquine (Brown et al., 2000).

Thus it is seen that lysine can be employed to build an intracellular DNA delivery system, which in addition to being efficient is also non-toxic.

Finally the biochemical roles and applications of lysine are summarised in Figure 2.

References

Aaregawa T (1999) bFGF increases regional myocardial blood flow and salvages myocardium in the infarcted border zone in a rabbit model of acute myocardial infarction. Angiology 50(6): 487–495.

Allison AC (1999) Squalene and squalane emulsions as adjuvants. Methods 19: 87–93.

Amante FH & Good MF (1997) Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. Parasite Immunol 19: 111–126.

Andreasen CB & Frank DE (1999) The effects of ascorbic acid on *in vitro* heterophil function. Avian Dis 43: 656–63.

Aoki M (2000) Angiogenesis induced by HGF in non infarcted myocardium and infarcted myocardium: upregulation of essential transcription factor for angiogenesis. Gene Ther 7: 417–427.

Appendini P & Hotchkiss JH (1999) Antimicrobial activity of a 14-residue peptide against *Escherichia coli* O157:H7. J Appl Microbiol 87: 750–756.

Ashcroft GS, Greenwell-Wild T, Horan MA, Wahl SM & Ferguson MW (1999a) Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. Am J Pathol 155: 1137–1146.

Ashcroft GS, Yang X, Glick AB, weinstein M, Lelterio JL, Mizel DE, Anzan OM, Greenwell-Wild T, Wahl SM, Deng C & Roberts AB (1999b) Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol 1: 260–266.

Bagarazzi ML, Boyer JD, Ugen KE, Javadian MA, Chattergoon M, Shah A, Bennett M, Ciccarelli R, Carrano R, Coney L & Weiner DB (1998) Safety and immunogenicity of HIV-1 DNA constructs in chimpanzees. Vaccine 16: 1836–1841.

Baichey G, Penkoya R & Deliiski T (1995) The treatment of slowly healing wounds with collagen and growth factors. Khururgiia (Sofiia) 48: 17–19.

Banker G & Goslin K (1991) Culturing Nerve Cells (pp. 65). MIT Press, Cambridge.

Barnett PV, Pullen L, Williams L & Doel TR (1996) International bank for foot-and-mouth disease vaccine: Assessment of Montanide ISA 25 and ISA 206, two commercially available oil adjuvants. Vaccine 14: 1187–1198.

Bellini AM, Vertuani G, Quaglio MP & Cavazzini G (1979) Bile acid derivatives with antimicrobial activity. Farmaco 34: 967–978.

Bennet CF, Chiang MY, Chan H et al. (1992) Cationic lipids enhance cellular uptake and activity of phosphothioate antisense oligonucleotides. Mol Pharmacol 41: 1023–1033.

Berger DP, Homann D & Oldstone MB (2000) Defining parameters for successful immunocytotherapy of persistent viral infection. Virology 266: 257–263.

Berns KI, Pinkerton TC, Thomas GF et al. (1975) Detection of adeno-associated virus (AAV)-specific nucleotide sequences in DNA isolated from latently infected Detroit 6 cells. Virology 68: 556–560.

Berstad AK, Andersen SR, Dalseg R, Dromtorp S, Holst J, Namork E, Wedege E & Haneberg B (2000) Inactivated meningococci and pertussis bacteria are immunogenic and act as mucosal adjuvants for a nasal inactivated influenza virus vaccine. Vaccin 18: 1910–1919.

Bibila TA & Robinson DK (1995) In pursuit of the optimal fed-batch process for monoclonal antibody production. Biotechnol Prog 11, 1–13.

Bibila TA, Glazomitsky K, Ranucci CS et al. (1994) Monoclonal antibody process development using medium concentrates. Biotechnol Prog 10: 87–96.

Bickford PC, Shukitt-Hale B & Joseph J (1999) Effects of aging on cerebellar noradrenergic function and motor learning: Nutritional interventions. Mech Ageing Dev 111: 141–154.

Blaszyk M & Holley RA (1998) Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. Int J Food Microbiol 39: 175–183.

Boraschi D & Tagliabue A (1999) Interleukin-1 and interleukin-1 fragments as vaccine adjuvants. Methods 19: 108–113.

Boyer JD, Chattergoon MA, Ugen KE, Shah A, Bennett M, Cohen A, Nyland S, Lacy KE, Bagatazzi ML, Higgins TJ, Baine Y, Ciccarelli RB, Ginsberg RS, MacGregor RR & Weiner DB (1999) Enhancement of cellular immune response in HIV-1 seropositive individuals: A DNA-based trial. Clin Immunol 90: 100–107.

Boyer JD, Ugen KE, Wang B, Agadjanyan M, Gilbert L, Bagarazzi ML, Chattergoon M, Frost P, Javadian A, Williams WV, Refaeli Y, Ciccarelli RB, McCallus D, Coney L & Weiner DB (1997) Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. Nat Med 3: 526–532.

Boyle JS, Silva A, Brady JL & Lew AM (1997) DNA immunization: Induction of higher avidity antibody and effect of route on T cell cytotoxicity. Proc Natl Acad Sci USA 94: 14626–14631.

Breitbart AS, Mason JM, Urmacher C, Barcia M, Grant RT, Pergolizzi RG & Grande PA (1999) Gene enhanced tissue engineering: Applications for wound healing using cultured dermal fibroblasts transduced retrovirally with PDGF-B gene. Ann Plast Surg 43: 632–639.

Brown MD, Schatzlein A, Brownlie A, Jack V, Wang W, Tetley L, Gray AI & Uchegbu IF (2000) Preliminary characterization of novel amino acid based polymeric vesicles as gene and drug delivery agents. Bioconjug Chem 11: 880–891.

Bushell ME, Bell SL, Scott MF, Spier RE, Wardell JN & Sanders PG (1994) Enhancement of monoclonal antibody yield by hybridoma fed-batch culture resulting in extended maintenance of viable cell populations. Biotechnol Bioeng 44: 1099–1106.

Buzoni-Gatel D, Lepage AC, Dimier-Poisson IH, Bout DT & Kasper LH (1997) Adoptive transfer of gut intraepithelial lymphocytes protects against murine infection with Toxoplasma gondii. J Immunol 158: 5883–5889.

Camilleri-Bruet S, Devez F, Tissier F, Ducruit V, Le Tourneau A, Diebold J, Audouin J & Molina T (2000) Quality control and sensitivity of polymerase chain reaction techniques for the assessment of immunoglobulin heavy chain gene rearrangements from fixed- and paraffin-embedded samples. Ann Diagn Pathol 4: 71–76.

Cerwenka A, Morgan TM & Dutton RW(1999) Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: Homing properties rather than initial frequencies are crucial. J Immunol 163: 5535–5543.

Chan CK, Senden T & Jans DA (2000) Supramolecular structure and nuclear targeting efficiency determine the enhancement of transfection by modified polylysines. Gene Ther 7: 1690–1697.

Chang AE & Shu S (1996) Current status of adoptive immunotherapy of cancer. Crit Rev Oncol Hematol 22: 213–28.

Chattergoon MA, Kim JJ, Yang JS, Robinson TM, Lee DJ, Dentchev T, Wilson DM, Ayyavoo V & Weiner DB (2000) Targeted antigen delivery to antigen-presenting cells including dendritic cells by engineered Fas-mediated apoptosis. Nat Biotechnol 18: 974–979.

Chen C, Ji H, Suh KW, Choti MA, Pardoll DM & Wu T (1999) Gene gun-mediated DNA vaccination induces antitumor immunity against human papillomavirus type 16 E7-expressing murine tumor metastases in the liver and lungs. Gene Ther 6:

1972–1981.
Chen LH, Boissonneault GA & Glauert HP (1988) Vitamin C, vitamin E and cancer (review). Anticancer Res 8: 739–748.
Cheung AK, Hoggan MD, Hauswirth WW et al. (1980) Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J Virol 33: 739–748.
Cho YW, Cho YN, Chung SH, Yoo G & Ko SW (1999) Water soluble chitin as wound healing accelerator. Biomaterials 20: 2139–2145.
Chua FK, Yap MG & Oh SK (1994) Hyper-stimulation of monoclonal antibody production by high osmolarity stress in eRDF medium. J Biotechnol 37: 265–75.
Chua FKF, Yap MGS & Oh SKW (1994) Hyperstimulation of monoclonal antibody production by high osmolarity stress in eRDF medium. J Biotechnol 37: 265–275.
Converse PJ, Haines VL, Wondimu A, Craig LE & Meyers WM (1995) Infection of SCID mice with Mycobacterium leprae and control with antigen-activated 'immune' human peripheral blood mononuclear cells. Infect Immun 63: 1047–54.
Correa R, Blanco B, Del Rio M, Victor V, Guayerbas N, Medina S & De la Fuente M (1999) Effect of a diet supplemented with thioproline on murine macrophage function in a model of premature ageing. Biofactors 10: 195–200.
Cournoyer D & Caskey CT (1993) Gene therapy of the immune system. Annu Rev Immunol 11: 297–329.
Cynober LA (1999) The use of alpha-ketoglutarate salts in clinical nutrition and metabolic care. Curr Opin Clin Nutr Metab Care 2: 33–37.
Datta D (2000) Essential amino acid lysine and its analogues support faster and qualitatively better healing of wound. www.pharma-transfer.com (Balantyne-Ross Publications; Invited submission).
Datta D & Kundu PK (1999) Do we have a healer molecule? In: Gordon Conference on Wound Repair.
Datta D, Kundu PK & Senthilkumar M (1997) Studies on high density culture of hybridoma cells (AE9D6) In-Vitro Artif Organs 21: 534 (Blackwell Publications).
Datta D, Kundu PK & Senthilkumar M (1997b) Effect of L-lysine on the growth and secretion of hybridoma cell (AE9D6). Biotechnology Technique 11: 617–620.
Datta D, Kundu PK, Siva Prasad N, Electricwalla SE & Varma R (1997c) Getting higher yields of monclonal antibodies in culture. Ind J Physiol Pharm 42: 155–171.
Datta D, Kundu PK, Siva Prasad N & Varma R (1997d) Monoclonal antibodies: A brief overview of high-density culture, downstream processing and recovery. Ind J Exp Biol 36: 125–135.
Datta D, Kundu PK, Biswas S, Dasgupta S, Bhinge A & Chandran V (2000a) Effect of cationic amino acid L-Lysine and its polymers on the growth and secretion of hybridoma cell line OKT3. Hybridoma 19: 339–346.
Datta D, Vidya C & Bhinge A (2000b) Cationic amino acid lysine and it's analogues (C-6 to C-10) in wound repair and angiogenesis. Proceedings of 1st World Congress in wound repair, Sept 2000, Sydney Australia.
Datta D, Kundu PK, Dasgupta S & Fulzele K (2001) Effect of addition of proton carriers in culture medium on growth and secretion of hybridoma cell line OKT3. Ind J Physiol Pharm (In press).
Davis HL, Suparto II, Weeratna RR, Jumintarto, Iskandriati DD, Chamzah SS, Ma'ruf AA, Nente CC, Pawitri DD, Krieg AM, Heriyanto, Smits W & Sajuthi DD (2000) CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine 18: 1920–1924.
Deshane J, Siegal GP, Wang M et al. (1997) Transductional efficiency and safety of an intraperitoneally delivered adenovirus encoding an anti-erbB-2 intracellular single-chain antibody for ovarian cancer therapy. Gynecol Oncol 64: 378–385.
Di Luigi L, Guidetti L, Pigozzi F, Baldari C, Casini A, Nordio M & Romanelli F (1999) Acute amino acids supplementation enhances pituitary responsiveness in athletes. Med Sci Sports Exerc 31: 1748–1754.
Dupuis M, McDonald DM & Ott G (1999) Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice. Vaccine 18: 434–439.
Egeter O, Mocikat R, Ghoreschi K, Dieckmann A & Rocken M (2000) Eradication of disseminated lymphomas with CpG-DNA activated T helper type 1 cells from nontransgenic mice. Cancer Res 60: 1515–1520.
Epand RM & Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biophys Acta 1462: 11–28.
Etchart NR, Buckland R, Liu MA, Wild TF & Kaiserlian D (1997) Class I-restricted induction by mucosal immunization with naked DNA encoding measles virus haemagglutinin. J Gen Virol 78: 1577–1580.
Ezaki T (2000) Antigen retrieval on formaldehyde-fixed paraffin sections: it's potential drawbacks and optimization for double immunostaining. Micron 31: 639–49.
Fan JJ & Chen JH (1999) Inhibition of aflatoxin-producing fungi by Welsh onion extracts. J Food Prot 62: 414–417.
Fan H, Lin Q, Morrissey GR & Khavari PA (1999) Immunization via hair follicles by topical application of naked DNA to normal skin. Nat Biotechnol 19: 870–872.
Fike RM, Pfohl JL, Epstein DA, Jayme DW & Weiss S (1991) Hybridoma growth and monoclonal antibody production in protein-free hybridoma medium. BioPharm 3: 26–29.
Flodin NW (1997) The metabolic roles, pharmacology, and toxicology of lysine. J Am Coll Nutr 16: 7–21.
Funfstuck R, Straube E, Schildbach O & Tietz U (1997) Prevention of reinfection by L-methionine in patients with recurrent urinary tract infection. Med Klin 92: 574–581.
Geissler M, Wands G, Gesien A, Monte S, Bellet D & Wands JR (1997) Genetic immunization with the free human chorionic gonadotropin beta subunit elicits cytotoxic T lymphocyte responses and protects against tumor formation in mice. Lab Investig 76: 859–871.
Gilboa E (1990) Retroviral gene transfer. Applications to human gene therapy. Prog Clin Biol Res 352: 301–311.
Glorioso JC, De Luca NA & Fink DJ (1995) Development and application of herpes simplex virus vectors for human gene therapy. Annu Rev Microbiol 49: 675–710.
Goodman DL, McDonnell JT, Nelson HS, Vaughan TR & Weber RW (1990). Chronic urticaria exacerbated by the antioxidant food preservatives, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). J Allergy Clin Immunol 86 (4 Pt 1): 570–575.
Goodson B, Ehrhardt A, Ng S, Nuss J, Johnson K, Giedlin M, Yamamoto R, Moos WH, Krebber A, Ladner M, Giacona MB, Vitt C & Winter J (1999) Characterization of novel antimicrobial peptoids. Antimicrob Agents Chemother 43: 1429–1434.
Grizard D & Barthomeuf C (1999) Non-digestible oligosaccharides used as prebiotic agents: Mode of production and beneficial effects on animal and human health. Reprod Nutr Dev 39: 563–88.
Grzybowski J, Oldak E & Janiak MK (1999) Local application of Granulocyte-colony stimulating factor, Granulocyte-macrophage colony stimulating factor and Epidermal growth factor in treatment of wounds. Postepy Hig Med Dosw 53: 75–86.
Gupta RK, Chang AC & Siber GR (1998) Biodegradable polymer microspheres as vaccine adjuvants and delivery systems. Dev

Biol Stand 92: 63–78.
Gustafsson A, Levitsky V, Zou JZ, Frisan T, Dalianis T, Ljungman P, Ringden O, Winiarski J, Ernberg I & Masucci MG (2000) Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. Blood 95: 807–814.
Ham RG & McKeehan WL (1979) Methods in enzymology, In: W Jakoby and IH Pastan (eds.), Cell Culture, Vol. LVIII, (p. 49), Academic Press Limited, London.
Hamajima K, Sasaki S, Fukushima J, Kaneko T, Xin KQ, Kudoh I & Okuda K (1998) Intranasal administration of HIV-DNA vaccine formulated with a polymer, carboxymethylcellulose, augments mucosal antibody production and cell-mediated immune response. Clin Immunol Immunopathol 88: 205–210.
Haneberg B, Dalseg R, Oftung F, Wedege E, Hoiby EA, Haugen IL, Holst J, Andersen SR, Aase A, Meyer Naess L, Michaelsen TE, Namork E & Haaheim LR (1998) Towards a nasal vaccine against meningococcal disease, and prospects for its use as a mucosal adjuvant. Dev Biol Stand 92: 127–133.
Hansen G, Yeung VP, Berry G, Umetsu DT & DeKruyff RH (2000) Vaccination with heat-killed Listeria as adjuvant reverses established allergen-induced airway hyperreactivity and inflammation: role of CD8+ T cells and IL-18. J Immunol 164: 223–30.
Hantschick M & Stosiek P (1998) Cold acetone fixation and methacrylate embedding. A suitable method for routine processing of bone marrow biopsies. Pathol Res Pract 194: 111–121.
Haynes BF (1996) HIV vaccines: Where we are and where we are going. Lancet 348: 933–937.
Heimbach J, Rieth S, Mohamedshah F, Slesinski R, Samuel-Fernando P, Sheehan T, Dickmann R & Borzelleca J (2000) Safety assessment of iron EDTA [sodium iron ($Fe^{(3+)}$) ethylenediaminetetraacetic acid]: Summary of toxicological, fortification and exposure data. Food Chem Toxicol 38: 99–111.
Hiller GW, Clark DS & Blanch HW (1994) Transient responses of hybridoma cells in continuous culture to step changes in amino acid and vitamin concentrations. Biotechnol Bioeng 44: 303–321.
Hirunpetcharat C & Good MF (1998) Deletion of Plasmodium berghei-specific $CD4^+$ T cells adoptively transferred into recipient mice after challenge with homologous parasite. Proc Natl Acad Sci USA 95: 1715–1720.
Hodinka RL, Jack-Wait K, Wannamaker N, Walden TP & Gilligan PH (1987) Comparative *in vitro* activity of LY146032 (daptomycin), a new lipopeptide antimicrobial. Eur J Clin Microbiol 6: 100–103.
Hong J, Oren Z & Shai Y (1999) Structure and organization of hemolytic and nonhemolytic diastereomers of antimicrobial peptides in membranes. Biochemistry 38: 16963–16973.
Inamori Y, Amino H, Tsuboi M, Yamaguchi S & Tsujibo H (1990) Biological activities of racemomycin-B, beta-lysine rich streptothricin antibiotic, the main component of Streptomyces lavendulae OP-2. Chem Pharm Bull (Tokyo) 38: 2296–2298.
Isner JM (1999) Manipulating angiogenesis against vascular diseases. Hosp Pract (Off Ed) 34: 69–74, 76, 79–80.
Ivashkin VT, Tel'nykh IuV, Kuznetsov EN & Kolokol'nikova OA (1999) Use of biologically active food additive cardiohels in the treatment of patients with ischemic heart disease. Klin Med (Mosk) 77: 34–36.
Jaschke E, Zabernigg A & Gattringer C (1999) Recombinant human Granulocyte-macrophage colony stimulating factor applied locally in low doses enhances healing and prevents recurrence of chronic venous ulcers. Int J Dermatol 38: 380–386.
Jenkins TC (2000) Feeding oleamide to lactating Jersey cows 1. Effects on lactation performance and milk fatty acid compositio. J Dairy Sci 83: 332–337.
Jennings R, Simms JR & Heath AW (1998) Adjuvants and delivery systems for viral vaccines-mechanisms and potential. Dev Biol Stand 92: 19–28.
Jia C, Chen B & Arnold F (1998) The effect of ultrapure hyaluronic acid with different molecular weights on the healing of porcine full thickness skin wound. Chung Kuo Hsiu Fu Chung Chien Wai Ko Tsa Chih 12: 197–200.
Jo EC, Park HJ, Kim D & Moon HM (1992) Step fortifications of nutrients in mammalian cell culture. Biotechnol Bioeng 42: 1218–1226.
Jo EC, Park HJ, Kim D & Moon HM (1993) Repeated fed-batch culture of hybridoma cells in nutrient-fortified high-density medium. Biotechnol Bioeng 42: 1229–1237.
Junienez PA & Rampy MA (1999) Keratinocyte growth factor – 2 accelerates wound healing in incisional wounds. J Surg Res 81: 238–242.
Karlsson S, Van Doren K, Schweiger SG et al. (1985) Stable gene transfer and tissue-specific expression of a human globin gene using adenoviral vectors. EMBO J 5: 2377–2385.
Kasper LH & Khan IA (1998) Antigen-specific $CD8^+$ T cells protect against lethal toxoplasmosis in mice infected with Neospora caninum. Infect Immun 66: 1554–1560.
Ker-hwa Ou S, Patterson PH (1997) A more efficient and economical approach for monoclonal Antibody production. J Immunol 209: 105–108.
Kim JJ, Nottingham LK, Tsai A, Lee DJ, Maguire HC, Oh J, Dentchev T, Manson KH, Wyand MS, Agadjanyan MG, Ugen KE & Weiner DB (1999) Antigen-specific humoral and cellular immune responses can be modulated in rhesus macaques through the use of IFN-gamma, IL-12, or IL-18 gene adjuvants. J Med Primatol 28: 214–223.
Kim JJ, Yang JS, Lee DJ, Wilson DM, Nottingham LK, Morrison L, Tsai A, Oh J, Dang K, Dentchev T, Agadjanyan MG, Sin JI, Chalian AA & Weiner DB (2000) Macrophage colony-stimulating factor can modulate immune responses and attract dendritic cells *in vivo*. Hum Gene Ther 11: 305–321.
Klavinskis LS, Barnfield C, Gao L & Parker S (1999) Intranasal immunization with plasmid DNA-lipid complexes elicits mucosal immunity in the female genital and rectal tracts. J Immunol 162: 243–262.
Kohler G & Milstein (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495–497.
Kohn DB, Anderson WF & Blaese MB (1989) Gene therapy for genetic diseases. Cancer Invest 7: 179–192.
Kolsarici N & Candogan K (1995) The effects of potassium sorbate and lactic acid on the shelf-life of vaccum-packed chicken meats. Poult Sci 74: 1884–1893.
Kornowski R et al. (2000) Delivery strategies to achieve therapeutic myocardial angiogenesis. Circulation 101: 454–458.
Kovarik J & Siegrist CA (1998) Optimization of vaccine responses in early life: The role of delivery systems and immunomodulators. Immunol Cell Biol 76: 222–236.
Kratz G, Back M, Amander C & Larun O (1998) Immobilised heparin accelerates the healing of human wounds *in vivo*. Scand J Plast Reconstr Surg hand Surg 32: 381–385.
Kreider RB (1999) Dietary supplements and the promotion of muscle growth with resistance exercise. Sports Med 27: 97–110.
Krishnan L, Dicaire CJ, Patel GB & Sprott GD (2000) Archaeosome vaccine adjuvants induce strong humoral, cell-mediated, and memory responses: comparison to conventional liposomes and alum. Infect Immun 68: 54–63.

Krup OC, Kroll I, Bose G & Falkenberg FW (1999) Cytokine depot formulations as adjuvants for tumor vaccines. Liposome-encapsulated IL-2 as a depot formulation. J Immunother 22: 525–538.

Kumarasinghe MP, Constantine SR & Hemamali RL (1997) Methanol as an alternative fixative for cytological smears. Malays J Pathol 19: 137–140.

Kun LE, Gajjar A, Muhlbauer M et al. (1995) Stereotactic injection of herpes simplex thymidine kinase vector producer cells (PA 317-G1Tk1SvNa.7) and intravenous ganciclovir for the treatment of progressive or recurrent primary supratentorial pediatric malignant brain tumors. Hum Gene Ther 6: 1231–1255.

Kundu PK, Bharmal SE, Siva Prasad N & Datta D (1999) Scaled up culture of stimulated hybridoma cells AE9D6. Indian Chemical Engineer 41: 113–116.

Kurokawa H, Park YS, Iijima S & Kobayashi T (1994) Growth characteristics in fed-batch culture of hybridoma cells with control of glucose and glutamine concentrations. Biotechnol Bioeng 44: 95–110.

Kyriahides TR, Tain JW & Bomstein P (1999) Accelerated wound healing in mice with a disruption of thrombospondin 2 gene. J Invest Dermatol 113: 782–787.

LaRocca P & Barker S (1996) Tissue culture surface treatments. The Cell/Line 6: 1–6.

Laham RJ et al. (1999) Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial. Circulation 100: 1865–1871.

Laham RJ et al. (2000) Intrapericardial delivery of FGF2 induces neo-vascularisation in a porcine model of chronic myocardial ischemia. J Pharmacol Exp Ther 292: 795–802.

Lai E & van Zanten JH (2001) Monitoring DNA/poly-L-lysine polyplex formation with time-resolved multiangle laser light scattering. Biophys J 80: 864–873.

Lansdown AB, Sampson B, Laupattarakasam P & Vultivirojana A (1997) Silver aids healing in the sterile skin wounds: Experimental studies in Laboratory rat. Br J Dermatol 137: 728–735.

Lee IH, Cho Y & Lehrer RI (1997) Styelins, broad-spectrum antimicrobial peptides from the solitary tunicate, Styela clava. Comp Biochem Physiol Biochem Mol Biol 118: 515–521.

Lee LY et al. (2000). Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for VEGF121. Ann Thorac Surg Jan 69: 14–23.

Lee GM & Palsson BO (1993) Stability of antibody productivity is improved when hybridoma cells are entrapped in calcium alginate beads. Biotechnol Bioeng 42: 1131–1135.

Levashina EA, Ohresser S, Bulet P, Reichhart JM, Hetru C & Hoffmann JA (1995) Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties. Eur J Biochem 233: 694–700.

Li J et al. (2000) PR39, a peptide regulator of angiogenesis. Nat Med 6: 49–55.

Li Q & Chang AE (1999) Adoptive T-cell immunotherapy of cancer. Cytokines Cell Mol Ther 5: 105–117

Li Q, Normolle DP, Sayre DM, Zeng X, Sun R, Jiang G, Redman BD & Chang AE (2000) Immunological effects of BCG as an adjuvant in autologous tumor vaccines. Clin Immunol 94: 64–72.

Liang JF & Kim SC (1999) Not only the nature of peptide but also the characteristics of cell membrane determine the antimicrobial mechanism of a peptide. J Pept Res 53: 518–522.

Liechty KW, Nesbit M, Herlyn M, Radu A, Adzick NS & Crombleholme TM (1999) Adenoviral-mediated overexpression of PDGF-B corrects ischemic impaired wound healing. J Invest Dermatol 113: 375–383.

Loomans HJ, Hahn BL, Li QQ, Phadnis SH & Sohnle PG (1998) Histidine-based zinc-binding sequences and the antimicrobial activity of calprotectin. J Infect Dis 177: 812–814.

Lu GZ, Thompson BG, Suresh MR & Gray MR (1995) Cultivation of hybridoma cells in an inclined bioreactor. Biotechnol Bioeng 45, 176–186.

Luengo JM, Revilla G, Lopez MJ, Villanueva JR & Martin JF (1980) Inhibition and repression of homocitrate synthase by lysine in Penicillium chrysogenum. Bacteriol 144: 869–876.

Maeda M, Murakami H, Ohta H & Tajima M (1992) Stimulation of IgM production in human-human hybridoma HB4C5 cells by chitosan. Biosci Biotechnol Biochem 56: 427–431.

Magovern CJ (1996) Direct *in vivo* gene transfer to canine myocardium using a replication deficient adenovirus vector. Ann Thorac Surg 62: 425–433.

Mahvi DM, Sondel PM, Yang NS et al. (1997) Phase I/IB study of immunization with autologous tumor cells transduced with the GM-CSF gene by particle-mediated transfer in patients with melanoma or sarcoma. Hum Gene Ther 8: 875–891.

Malinda KM, Sidhu GS, Mani H, Banaudha K, Maheshwari RK, Goldstein AL & Kleinman HK (1999) Thymosin beta 4 accelerates wound healing. J Invest Dermatol 113: 364–368.

Maneta-Peyret L, Compere P, Moreau P, Goffinet G & Cassagne C (1999) Immunocytochemistry of lipids: Chemical fixatives have dramatic effects on the preservation of tissue lipids. Histochem J 31: 541–547.

Markiewicz MA & Gajewski TF (1999) The immune system as anti-tumor sentinel:molecular requirements for an anti-tumor immune response. Crit Rev Oncog 10: 247–260.

Martens DE, De Gooijer CD, Beuvery EC & Tramper J (1992) Effect of serum concentration on hybridoma viable cell density and production of monoclonal antibodies in CSTRs and on shear sensitivity in air-loop reactors. Biotechnol Bioeng 39: 891–897.

Marty HH & Risau W (1999) Angiogenesis in Ischemic disease. Thromb Haemost 82 (Suppl 1): 44–52.

Masalova OV, Shepelev AV, Atanadze SN, Parnes ZN, Romanova VS, Vol'pina OM, Semiletov IuA & Kushch AA (1999) Immunostimulating effect of water-soluble fullerene derivatives-perspective adjuvants for a new generation of vaccine. Dokl Akad Nauk 369: 411–413.

Massa LF & Arana-Chavez VE (2000) Ultrastructural preservation of rat embryonic dental tissues after rapid fixation and dehydration under microwave irradiation. Eur J Oral Sci 108: 74–77.

Matsuda S, Iwata H, Se N & Ikada Y (1999) Bioadhesion of gelatin films crosslinked with gluteraldehyde. J Biomed Mater Res 45: 20–27.

Maxwell AJ, Anderson BE & Cooke JP (2000) Nutritional therapy for peripheral arterial disease: A double-blind, placebo-controlled, randomized trial of HeartBar. Vasc Med 5: 11–9.

McKeehan WL & Ham RG (1976) Stimulation of clonal growth of normal fibroblasts with substrata coated with basic polymers. J Cell Biol 71: 727–734.

McKeehan WL (1984) Methods for Preparation of Media, Supplements, and Substrata for Serum-free Animal Cell Culture (p. 209), A.R. Liss, New York.

Medina E, Talay SR, Chhatwal GS & Guzman CA (1998) Fibronectin-binding protein I of Streptococcus pyogenes is a promising adjuvant for antigens delivered by mucosal route. Eur J Immunol 28: 1069–1077.

Meng WC, Leung KL, Ho RL, Leung TW & Lau WY (1999) Prospective randomized control study on the effect of branched-chain amino acids in patients with liver resection for hepatocellular carcinoma. Aust N Z J Surg 69: 811–815.

Miller AD (1990) Retrovirus packaging cells. Hum Gene Ther 1: 5–14.

Montville TJ & Chen Y (1998) Mechanistic action of pediocin and nisin: Recent progress and unresolved questions. Am J Contact Dermat Appl Microbiol Biotechnol 50: 511–519.

Moore GE & Hood DB (1993) Modified RPMI 1640 culture medium. In Vitro Cell Dev Biol 29A: 265–267.

Mor A, Hani K & Nicolas P (1994) The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. J Biol Chem 269: 31635–31641.

Morein B, Villacres-Eriksson M, Sjolander A & Bengtsson KL (1996) Novel adjuvants and vaccine delivery systems. Vet Immunol Immunopathol 54: 373–384.

Moulin V, Lawny F, Barritault D & Caruelle JT (1998) Platelet release treatment improves skin healing in diabetic rats through endogenous growth factor secretion. Cell Mol Biol (Noisy-legrand) 44: 961–971.

Mowad CM (2000) Allergic contact dermatitis caused by parabens: 2 case reports and a review. Am J Contact Dermat 11: 53–56.

Oikawa S, Nishino K, Oikawa S, Inoue S, Mizutani T & Kawanishi S (1998) Oxidative DNA damage and apoptosis induced by metabolites of butylated hydroxytoluene. Biochem Pharmacol 56: 361–370.

Mrsny RJ (1998) Adjuvants and delivery issues related to immunization: A survey of the recent patent literature. J Drug Target 6: 243–249.

Murray K, Gull K & Dickson AJ (1996) Dichloroacetate increases cell and antibody yield in batch cultures of a hybridoma cell line. Biotechnol Bioeng 49: 377–382.

Nguyen DM, Spitz FR, Yen N et al. (1996) Gene therapy for lung cancer: Enhancement of tumor suppression by a combination of sequential systematic cisplatin and adenovirus-mediated p53 gene transfer. J Thorac Cardiovasc Surg 112: 1372–1376.

Oh JE & Lee KH (1999) Synthesis of novel unnatural amino acid as a building block and its incorporation into an antimicrobial peptide. Bioorg Med Chem 7: 2985–2990.

Oh JE, Hong SY & Lee KH (1999) The comparison of characteristics between membrane-active antifungal peptide and its pseudopeptides. Bioorg Med Chem 7: 2509–2515.

Onishi JC, Milligan JA, Basilio A, Bergstrom J, Curotto J, Huang L, Meinz M, Nallin-Omstead M, Pelaez F, Rew D, Salvatore M, Thompson J, Vicente F & Kurtz MB (1997) Antimicrobial activity of viridiofungins. J Antibiot (Tokyo) 50: 334–338.

Ono I, Pateshita T & Inoue M (1999) Effects of a collagen matrix containing basic fibroblast growth factor on wound contraction. J Biomed Mater Res 48: 621–630.

Oros G, Ujvary I & Nachman RJ (1999) Antimicrobial activity of o-carboranylalanine. Amino Acids 17: 357–368.

Osanto S, Brouwenstyn N, Vaessen N et al. (1993) Immunization with interleukin-2 transfected melanoma cells. A phase I-II study in patients with metastatic melanoma. Hum Gene Ther 4: 323–330.

Ozturk SS & Palsson BO (1991) Physiological changes during the adaptation of hybridoma cells to low serum and serum-free media. Biotechnol Bioeng 37: 25–46

Pandit A, Ashar R & Feldman D (1999) The effect of TGF-b delivered through a collagen scaffold on wound healing. J Invest Surg 12: 89–100.

Park CJ, Park CB, Hong SS, Lee HS, Lee SY & Kim SC (2000) Characterization and cDNA cloning of two glycine- and histidine-rich antimicrobial peptides from the roots of Shepherd's purse, Capsella bursa-pastoris. Plant Mol Biol 44: 187–197.

Parke DV & Lewis DF (1992) Safety aspects of food preservatives. Food Addit Contam 9: 561–577.

Pedersen KL, Pedersen SN, Christiansen LB, Korsgaard B & Bjerregaard P (2000) The preservatives ethyl-, propyl- and butylparaben are oestrogenic in an *in vivo* fish assay. Pharmacol Toxicol 86: 110–113.

Piper PW (1999) Yeast superoxide dismutase mutants reveal a prooxidant action of weak organic acid food preservatives. Free Radic Biol Med 27: 1219–1227.

Podsakoff G, Wong Jr KK & Chatterjee S (1994) Efficient gene transfer into nondividing cells by adeno-associated virus-based vectors. J Virol 68: 5656–5666.

Ramshaw IA, Fordham SA, Bernard CC, Maguire D, Cowden WB & Willenborg DO (1997) DNA vaccines for the treatment of autoimmune disease. Immunol Cell Biol 75: 409–413.

Rangell LK & Keller GA (2000) Application of microwave technology to the processing and immunolabeling of plastic-embedded and cryosections. J Histochem Cytochem 48: 1153–1160.

Rasik AM, Raghubir R, Gupta A, Shukla A, Dubey MP, Srivastava S, Jain HK & Kulshrutha DK (1999) Healing potential of Calotropis procera on dermal wounds in Guinea pigs. J Ethnopharmacol 68: 261–266.

Ray EC, Landis ME & Miller VM (1999) Effects of dietary L-arginine on the reactivity of canine coronary arteries. Vasc Med 4: 211–217.

Robinson PH, Chalupa W, Sniffen CJ, Julien WE, Sato H, Fujieda T, Watanabe K & Suzuki H (1999) Influence of postruminal supplementation of methionine and lysine, isoleucine, or all three amino acids on intake and chewing behavior, ruminal fermentation, and milk and milk component production. J Anim Sci 77: 2781–2792.

Rodolfo M & Colombo MP (1999) Interleukin-12 as an adjuvant for cancer immunotherapy. Methods 19: 114–120.

Ropert C, Malvy C & Couvreur P (1993) Inhibition of the Friend retrovirus by antisense oligonucleotides encapsulated in liposomes: mechanism of action. Pharm Res 10: 1427–1433.

Rosenberg SA, Aebersold P, Cornetta K et al. (1990) Gene transfer into humans: immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med 323: 570–578.

Ruszczak Z & Schwartz RA (1999) Collagen uses in dermatology – An update. J Dermatol 199: 285–289.

Ryan EJ, McNeela E, Murphy GA, Stewart H, O'Hagan D, Pizza M, Rappuoli R & Mills KH (1999) Mutants of Escherichia coli heat-labile toxin act as effective mucosal adjuvants for nasal delivery of an acellular pertussis vaccine: Differential effects of the nontoxic AB complex and enzyme activity on Th1 and Th2 cells. Infect Immun 67: 6270–6280.

Sadelain M & Latouche JB (2000) Induction of human cytotoxic T lymphocytes by artificial antigen-presenting cells. Nat Biotechnol 18: 405–409.

Sanfeliu A, Cairo JJ, Casas C, Sola C & Godia F (1996) Analysis of nutritional factors and physical conditions affecting growth and monoclonal antibody production of the hybridoma KB-26.5 cell line. Biotechnol Prog 12: 209–216.

Santin AD, Hermonat PL, Ravaggi A, Bellone S, Cowan C, Korourian S, Pecorelli S, Cannon MJ & Parham GP (2000) Development, characterization and distribution of adoptively transferred peripheral blood lymphocytes primed by human papillomavirus 18 E7-pulsed autologous dendritic cells in a patient with metastatic adenocarcinoma of the uterine cervix. Eur J Gynaecol Oncol 21: 17–23.

Scannell AG, Hill C, Buckley DJ & Arendt EK (1997) Determination of the influence of organic acids and nisin on shelf-life and microbiological safety aspects of fresh pork sausage. J Appl

Microbiol 83: 407–412.
Schlecht S, Wiesmuller KH, Jung G & Bessler WG (1993) Lipopeptides as natural adjuvants for vaccines from Gram-negative bacteria. Naturwissenschaften 80: 9–17.
Schrijver IA, Melief MJ, van Meurs M, Companjen AR & Laman JD (2000) Pararosaniline fixation for detection of co-stimulatory molecules, cytokines, and specific antibody. J Histochem Cytochem Jan 48(1): 95–103.
Schwarzenberger P, Huang W, Oliver P, Osidipe T, Theodossiou C & Kolls JK (2001) Poly-L-lysine-based molecular conjugate vectors: a high efficiency gene transfer system for human progenitor and leukemia cells. Am J Med Sci 321: 129–136.
Seo SH, Pei J, Briles WE, Dzielawa J & Collisson EW (2000) Adoptive transfer of infectious bronchitis virus primed alphabeta T cells bearing CD8 antigen protects chicks from acute infection. Virology 269: 183–189.
Serth J, Kuczyk MA, Paeslack U, Lichtinghagen R & Jonas U (2000) Quantitation of DNA extracted after micropreparation of cells from frozen and formalin fixed tissue sections. Am J Pathol 156: 1189–1196.
Shedlock DJ & Weiner DB (2000) DNA vaccination: Antigen presentation and the induction of immunity. J Leukocyte Biol 68: 793–806.
Sheu BC, Hsu SM, Ho HN, Lin RH & Huang SC (1999) Tumor immunology-when a cancer cell meets the immune cells. J Formos Med Assoc 98: 730–735.
Shibutani M, Uneyama C, Miyazaki K, Toyoda K & Hirose M (2000) Methacarn fixation: A novel tool for analysis of gene expressions in paraffin-embedded tissue specimens. Lab Invest 80: 199–208.
Sidhu GS, Singh AK, Banaudha KK, Gaddipati JP, Patuaik GK & Maheshwari RK (1999a) Arnebin-1 accelerates normal and hydrocortisone induced impaired wound healing. J Invest Dermatol 113: 773–781.
Sidhu GS, Mani H, Gaddipati JP, Singh AK, Seth P, Banaudha KK, Patnaik GK & Maheshwari RK (1999b) Curcumin enhances wound healing in streptozotocin induced diabetic rats and genetically diabetic mice. Wound Repair Regen 7: 362–374.
Sin JI, Bagarazzi M, Pachuk C & Weiner DB (1999) DNA priming-protein boosting enhances both antigen-specific antibody and Th1-type cellular immune responses in a murine herpes simplex virus-2 gD vaccine model. DNA Cell Biol 18: 771–779.
Singh M & O'Hagan D (1999) Advances in vaccine adjuvants. Nat Biotechnol 17: 1075–1081.
Soda H, Koda K, Yasutomi J, Oda K, Takiguchi N, Saito N & Nakajima N (1999) Adoptive immunotherapy for advanced cancer patients using *in vitro* activated cytotoxic T lymphocytes. J Surg Oncol 72: 211–217
Sreter L (1999) The role of nutrition in the pathogenesis and prevention of oncologic diseases. Orv Hetil 140: 2275–2283.
Stanchev M, Tabakova S, Videnov G, Golovinsky E & Jung G (1999) Synthesis and antimicrobial activity *in vitro* of new amino acids and peptides containing thiazole and oxazole moieties. Arch Pharm (Weinheim) 332: 297–304.
Sugahara T, Shirahata S, Akiyohsi K, Isobe T, Okuyama T & Murakami H (1991) Immunoglobulin production stimulating factor-IIa (IPSF IIα) is a glyceraldehyde-3-phosphate dehydrogenase like protein. Cytotechnology 6: 115–120.
Sugahara T, Sasaki T & Murakami H (1994) Enhancement of immunoglobulin productivity of human-human hybridoma HB4C5 cells by basic proteins and poly-basic amino acids. Biosci Biotech Biochem 58: 2212–2214.
Sugahara T, Furutani H & Sasaki T (1997) Alcohol dehydrogenase-I from horse liver stimulates immunoglobulin production by human hybridoma and human peripheral blood lymphocytes. Mol Cell Biochem 173: 113–9.
Sugahara T, Shimizu S, Abiru M, Matsuoka S & Sasaki T (1998) A novel function of enolase from rabbit muscle; an immunoglobulin production stimulating factor. Biochim Biophys Acta 1380: 163–176.
Takahashi K, Onodera K & Akiba Y (1999) Effect of dietary xylitol on growth and inflammatory responses in immune stimulated chickens. Br Poult Sci 40: 552–4.
Talwar M, Moyana TN, Bharadwaj B & Tan LK (1996) The effect of synthetic analogue of PGE2 on wound healing in rats. Ann Clin Lab Sci 26: 451–457.
Tam JP, Lu YA, Yang JL & Chiu KW (1999) An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. Proc Natl Acad Sci USA 96: 8913–8918.
Tascon RE, Soares CS, Ragno S, Stavropoulos E, Hirst EM & Colston MJ (2000) Mycobacterium tuberculosis-activated dendritic cells induce protective immunity in mice. Immunology 99: 473–480.
Tawil NJ, Connors D, Gies D, Bennet S, Gruskin E & Mustoe T (1999) Stimulation of wound healing by positively charged dextran beads depends upon clustering of beads and cells in close proximity to the wound. Wound Repair Regen 7: 389–399.
Temin HM (1986) Retrovirus vectors for gene transfer: Efficient integration into and expression of exogenous DNA in vertebrate cell genomes. In: R Kucherlapati (ed.), Gene Transfer (pp. 144–187) Plenum Press, New York.
Thierry AR & Dritschilo A (1992) Intracellular availability of unmodified, phosphorothioates and liposomal encapsulated oligodeoxynucleotides for antisense activity. Nucleic Acids Res 20: 5691–5698.
Thierry AR, Rahman A & Dritschilo A (1993) Overcoming multidrug resistance in human tumor cells using free and liposomally encapsulated antisense oligodeoxynucleotides. Biochem Biophys Res Commun 190: 952–960.
Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, Bender A, Maczek C, Schreiner D, von den Driesch P, Brocker EB, Steinman RM, Enk A, Kampgen E & Schuler G (1999) Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 190: 1669–1678.
Tjomsland O (1999) Transmyocardial laser treatment – A new treatment modality in pronounced angina pectoris. Tidsskr Nor Laegeforen 119: 3597–3601.
Toes RE, Offringa R, Feltkamp MC, Visseren MJ, Schoenberger SP, Melief CJ & Kast WM (1994) Tumor rejection antigens and tumor specific cytotoxic T lymphocytes. Behring Inst Mitt 94: 72–86.
Tomita S et al. (1999) Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 100 (Suppl II): 247–256.
Trengove NJ, Stacey MC, Mac Auley S, Bennet N, Gibson J, Burslem F, Murphy G & Schultz G (1999) Analysis of acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen 7: 442–452.
Tripp MW, Hoagland TA, Dahl GE, Kimrey AS & Zinn SA (1998) Methionine and somatotropin supplementation in growing beef cattle. J Anim Sci 76: 1197–203.
Ulrich JT & Myers KR (1995) Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. Pharm Biotechnol 6: 495–524.
Van Kraaij C, Breukink E, Noordermeer MA, Demel RA, Siezen RJ,

Kuipers OP & De Kruijff B (1998) Pore formation by nisin involves translocation of its C-terminal part across the membrane. Biochemistry 37: 16033–16040.

Van Slooten ML, Storm G, Zoephel A, Kupcu Z, Boerman O, Crommelin DJ, Wagner E & Kircheis R (2000) Liposomes containing interferon-gamma as adjuvant in tumor cell vaccines. Pharm Res 17: 42–48.

Vogel FR (1998) Adjuvants in perspective. Dev Biol Stand 92: 241–248.

Voigt A & Zintl F (1999) Hybridoma cell growth and anti-neuroblastoma monoclonal antibody production in spinner flasks using a protein-free medium with microcarriers. J Biotechnol 68: 213–226.

Von Hunolstein C, Teloni R, Mariotti S, Recchia S, Orefici G & Nisini R (2000) Synthetic oligodeoxynucleotide containing CpG motif induces an anti-polysaccharide type 1-like immune response after immunization of mice with haemophilus influenzae type b conjugate vaccine. Int Immunol 12: 295–303.

Wang B, Ugen KE, Srikantan V, Agadjanyan MG, Dang K, Refaeli Y, Sato A, Boyer J, Williams WV & Weiner DB (1993) Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc Natl Acad Sci USA 90: 4156–4160.

Wang B, Boyer JD, Srikantan V, Coney L, Carrano R, Phan C, Merva M, Dang K, Agadjanyan MG, Ugen KE, Williams MV & Weiner DB (1993a) DNA inoculation induces neutralizing immune responses against human immunodeficiency virus type 1 in mice and non-human primates. DNA Cell Biol 12: 799–805

Wang B, Dang K, Agadjanyan MG, Srikantan V, Li F, Ugen KE, Boyer J, Merva M, Williams WV & Weiner DB (1997) Mucosal immunization with a DNA vaccine induces immune responses against HIV-1 at a mucosal site. Vaccine 15: 821–825.

Wang S, Liu X, Fisher K, Smith JG, Chen F, Tobery TW, Ulmer JB, Evans RK & Caulfield MJ (2000) Enhanced type I immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminum phosphate. Vaccine 18: 1227–1235.

Weber J (1996) Distinguishing between response to HIV vaccine and response to HIV. Lancet 350: 230–231.

Woodworth JC, Goodband RD, Nelssen JL, Tokach MD & Musser RE (2000) Added dietary pyridoxine, but not thiamin, improves weanling pig growth performance. J Anim Sci 78: 88–93.

Xie L & Wang DIC (1994a) Stoichiometric analysis of animal cell growth and it's application in medium design. Biotechnol Bioeng 43: 1164–1174.

Xie L & Wang DIC (1994b) Fed-batch cultivation of animal cells using different design concepts and feeding strategies. Biotechnol Bioeng 43: 1175–1189.

Yamada M, Lewis JA & Grodzicker T (1985) Overproduction of the protein product of a nonselected foreign gene carried by an adenovirus vector. Proc Natl Acad Sci USA 82: 3597–3571.

Yamakawa K, Karasawa T, Ohta T, Hayashi H & Nakamura S (1998) Inhibition of enhanced toxin production by Clostridium difficle in biotin-limited conditions. J Med Microbiol 47: 767–771.

Yavin E & Yavin Z (1974) Attachment and culture of dissociated cells from rat embryo cerebral hemispheres on polylysine-coated surface. J Cell Biol 62: 540–546.

Zhang H, Gu X, Wang Q & Becker JM (1993) Preliminary experiments on the mode of action of oxalysine on Candida albicans and C. parapsilosis. Wei Sheng Wu Hsueh Pao 33: 411–417.

Zhou J, Song Y & Wang Y (1997) The study of epidermal growth factor in acceleration of skin wound healing. Chung Kuo HsinFu Chung Chien Wai Ko Tsa Chih 11: 267–268.