Chapter II

Literature Review

Psoriasis

Psoriasis is a common, chronic, inflammatory skin disorder, characterized by the appearance of whitish or silvery laminated scales on slightly raised, well marginated, reddish patches. Males and females are affected equally. Prevalence is between 0.5 and 3 % of general population (Barker, 1991).

Henseler and Christophers (1985) have segregated psoriatic patients into two groups based on age of onset, clinical course and genetic background. Type I psoriasis occurring at 16-22 years of age tends to be more severe, resistant to therapy, associated with a family history of the disease, and to have a poorer prognosis. Type 2 psoriasis occurring at 57-60 years of age is usually less severe and without family history.

Pathogenesis of psoriasis

The characteristic change is the markedly shortened turnover rate of the epidermal cells. Instead of the normal 28 days from cell division in the basal layers until the cell is shed from the stratum corneum, it takes only 3 to 4 days for this to occur in psoriasis. The histopathological changes include parakeratosis (retention of nuclei in cells in the keratin layer), hyperkeratosis (increased thickness of the keratin layer), hypogranulosis (loss of the granular layer), elongation of the epidermal rete ridges, pustules with surrounding intercellular edema (spongiform pustules), and papillomatosis (increased height of the dermal papillary pegs) with thinning of the suprapapillary epidermis. There is an inflammatory infiltrate in the upper dermis and proliferation of small blood vessels in the papillae (vascular ectasia). Mitotic figures are seen in the bottom three cell layers of the epidermis rather than just in the basal layer (Gennaro, 1995).

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The mechanisms proposed for psoriasis are complex and not well established, but immunological factors are clearly involved (Weinstein and Krueger, 1993). Epidermal keratinocytes are activated, proliferate and release cytokines. Their activation is partly antigen-dependent since lesional keratinocytes express HLA-DR, a protein that presents antigen to helper T-cells. These cells also express intercellular adhesion molecule-1 (ICAM-1), which is specifically involved in lymphocyte adherence to antigen-presenting cells. In addition, the epidermis contains predominantly CD8 suppressor-cytotoxic lymphocytes, (reversing the normal CD4/CD8 ratio), whereas the dermis has mainly CD4 helper T-cell subsets (similar to circulating lymphocytes) and interleukin-2 receptor positive (activated) T-cells.

Thus, it seems that keratinocytes and lymphocytes are activated along a pathway that results in their secondary proliferation and biochemical activation through common cytokine pathways (involving molecules such as interleukin-1, -6 and -8, interferon and granulocyte-macrophage colony stimulating factors). Finally, the reversal of normal CD4/CD8 ratios in the epidermis suggests the possibility that psoriasis is an autoimmune disease of autoreactive CD8 T-cells (Weller, 1996).

Barker (1991) proposed a model of the pathophysiological process of psoriasis which highlights the interrelationship between epidermal hyperproliferation and inflammation (Figure 1). Central to the model are interactions between T lymphocytes and keratinocytes, and the close spatial relation between the epidermis and dermal vascular endothelium. Initiation of pathways at points illustrated by trigger 1 and trigger 2 would explain both site (i.e., skin) specificity of psoriasis and the importance of genetic factors. Because early psoriatic changes occur in advancing lesions before the accumulation of inflammatory cells, the initial defect resides within the epidermis (trigger 1).

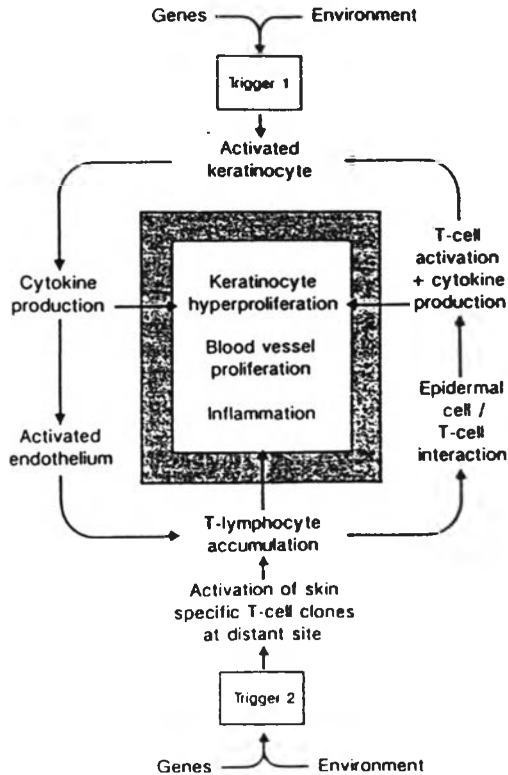

Figure 1. The pathophysiological mechanisms responsible for psoriasis (From Barker, 1991).

Clinical features

Psoriasis may present in various clinical forms, which may affect the same individual at different times. All the clinical variants have the important hallmarks of erythema, thickening, and scaling. Chronic plaque psoriasis is most common, while guttate, erythrodermic, and pustular psoriasis are less common variants (Weller, 1996; Weinstein and Krueger, 1993).

Chronic plaque psoriasis presents with the classical psoriatic lesions - well circumscribed, thickened, red, scaly plaques – typically on the elbows, knees, sacrum, buttocks, and scalp. Lesions may also develop at sites of trauma (Koebner phenomenon). Often nails are affected and, less commonly, the hands, feet, and face.

Sebopsoriasis has features which overlap with seborrhoeic dermatitis; facial and scalp involvement is more pronounced.

Guttate psoriasis presents as a sudden eruption of small psoriatic lesions on the trunk and limbs. It is often preceded by a viral or bacterial infection (especially of the upper respiratory tract).

Erythrodermic psoriasis presents as a non-specific erythema, which may be extensive and severe. It usually develops in chronic forms of psoriasis and may associated with serious systemic illness.

Pustular psoriasis may be generalized or localized. The generalized form is typically an acute serious illness with malaise, fever, and other systemic symptoms, often including metabolic dysfunction, such as hypocalcaemia. There may or may not be a past history of psoriasis and it may present in pregnancy. The localized form is usually confined to the palms and soles of middle-aged patients and lacks the systemic features. It may be very resistant to treatment.

Unstable psoriasis. Chronic stable psoriasis may become unstable at times. This is heralded by increasing erythema, burning discomfort or itch, and may be idiopathic or resulted from over-aggressive therapy or drugs given for other conditions (e.g., lithium, chloroquine, or non-steroidal anti-inflammatory drugs).

Non-cutaneous features. Psoriasis may be associated with disorders of organ systems other than the skin, such as arthritis. Psoriatic arthritis may affect any joint but is most typical in the hands and the lower spine.

PTU in the treatment of psoriasis

Propylthiouracil (PTU) is an antithyroid thioureylene which impairs thyroid hormone synthesis by binding to the T3 receptor (T3R). This receptor belongs to the family of steroid receptors and includes retinoid and vitamin D receptors. Since PTU may have modulatory activities on T lymphocytes such as inhibition of IL-2 production, it can additionally act as a free radical scavenger and have an antiproliferative activity (Elias, Goodman, and Rohan, 1993a; Elias, Goodman, and Rohan, 1993b; Elias et al., 1995).

Therefore, the clinical use of thioureylenes in psoriasis has been evaluated. In patients with psoriasis treated orally with PTU, a significant improvement in the Psoriasis Area and Severity Index (PASI) was observed after 2-3 months. Possible side effects of PTU treatment are hypothyroidism and leukopenia. However, only two of 15 patients treated with moderate dose PTU (300 mg/day) showed evidence of mild hypothyroidism. Neither of the two patients became clinically hypothyroid. In a different study, topical application of 5% PTU lotion (PTU was dissolved in propylene glycol and mixed with hydrophilic petrolatum) to patients with plaque psoriasis resulted in a significant improvement of the lesions (Elias et al., 1994). None of the patients receiving topical PTU developed clinical hypothyroidism or elevation of serum thyroid-stimulating hormone (TSH) levels. Although these data are encouraging, further clinical trials are required to evaluate the employment of PTU for the treatment of psoriasis. A further interest is the development of a suitable formulation for topical use in psoriatic therapy (Beissert and Luger, 1999).

Liposomes

(Betageri, Jenkins and Parsons, 1993; New, 1990; Stanzl, 1999; Sharma, 1997)

Liposomes are lipid vesicles composed of one or more lipid bilayers surrounding an equal number of aqueous compartments. The membrane components of liposomes have usually been phospholipids, particularly phosphatidylcholines (PC). Liposomes form spontaneously when the lipids are dispersed in aqueous media, giving rise to a population of vesicles which may range in size from tens of nanometers to tens of microns in diameter. Liposomes are classified by their size and number of bilayers into multilamellar vesicles (MLVs; several bilayers, size 0.1-20 μm), small unilamellar vesicles (SUVs; single bilayer, size 0.01-0.1 μm); and large unilamellar vesicles (LUVs; single bilayer, size 0.1-1 μm). Drugs with various lipophilicities can be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous volume or at the bilayer interface (Figure 2).

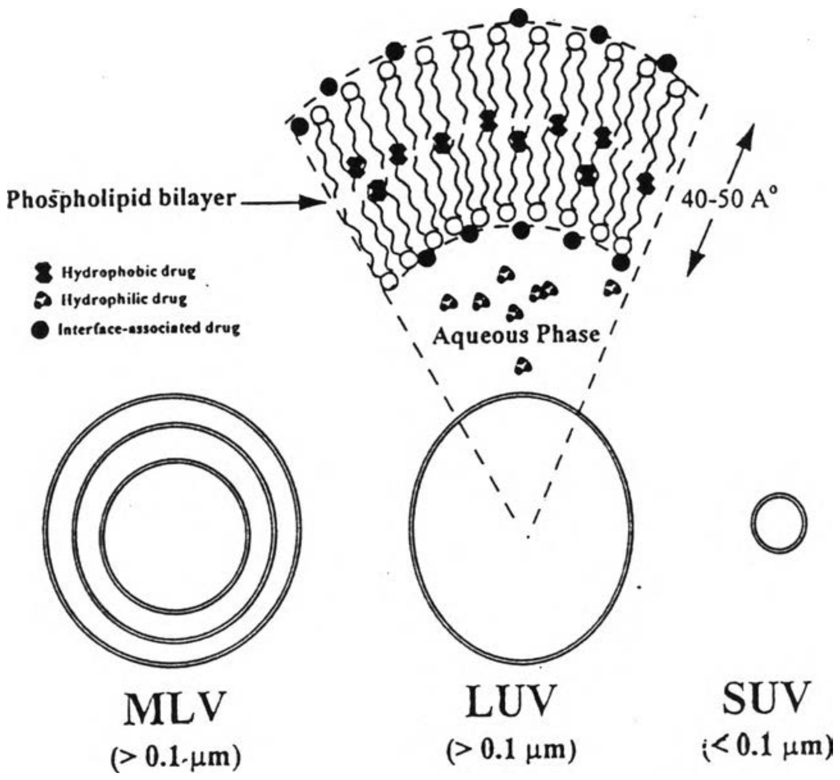

Figure 2. Types of liposomes depending on size and number of lamellae (From Sharma, 1997).

Phosphatidyl moiety	Headgroup	Common name Phosphatidyl	abbreviation
	$O-CH_2-CH_2-N^{+}(Me)_3$	choline	PC
	$O-CH_2-CH_2-NH_3^{+}$	ethanolamine	PE
	$O-CH(NH_3^{+})COO^{-}$	serine	PS
	$O-CH_2-CH(OH)-CH_2(OH)$	glycerol	PG
	O-H	acid	PA
	(inositol ring: OH, OH, OH, HO, OH)	inositol	PI

Figure 3. Chemical structure of some common phospholipids (From New, 1990).

Materials used in the preparation of liposomes

(Betageri, Jenkins, and Parsons, 1993; New, 1990; Philippot and Schuber, 1995; Weiner, Martin, and Riaz, 1989)

Neutral phospholipids

Liposomes can be prepared from a variety of lipids and lipid mixtures. Phospholipids are often used especially phosphatidylcholines, which are amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains with a hydrophilic polar head group (Figure 3). Molecules of phosphatidylcholines are not soluble in water in accepted sense, and in aqueous media they align themselves closely, in planar bilayer sheets, in order to minimize the unfavorable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets fold on themselves to form closed vesicles. Phosphatidylcholines contrast markedly with other amphipathic molecules (detergents, lysolecithin) in that bilayer sheets are formed in preference to micellar structures because the double fatty acid chains give the molecule an overall tubular shape, more suitable for aggregation in planar sheets compared with detergents with a polar head and single chain, whose conical shape fits into a spherical micellar structure (Figure 4). Phosphatidylcholines, also known as lecithin, can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean, but less readily from bovine heart and spinal cord. They are often used as the principal phospholipid in liposomes for a wide range of applications, both because of their low cost relative to other phospholipids, and their neutral charge and chemical inertness. Lecithin from natural sources is in fact a mixture of phosphatidylcholines, each with chains of different length and varying degrees of unsaturation. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contains a higher proportion of fully saturated chains.

In addition to phosphatidylcholine, neutral lipid bilayers may be composed of sphingomyelin or alkyl ether lecithin analogues. Replacement of ester bonds by ether

linkages increases the resistance of such lipids to hydrolysis, while apparently not greatly affecting the physical properties of the membranes. The other neutral phospholipid found commonly in natural membranes is phosphatidylethanolamine (PE). Possessing an unsubstituted quaternary ammonium group, which is protonated at neutral pH, this lipid differs from lecithin in two respects: first, its headgroup is smaller than the bulky phosphocholine of lecithin; second, it is able to take part in hydrogen bonding interactions with its neighbors in the membrane. Because of its small headgroup, PE can readily adopt a non-bilayer configuration in which the molecules form long cylindrical structures with the headgroups directed towards the center (Figure 4), known as the hexagonal (H_{II}) phase. In nature, the acyl chains of PE often vary considerably in length, and can be highly unsaturated, suggesting the possibility that PE may be involved in non-bilayer organization of membrane lipids in biological systems. PE is used mainly in construction of pH-sensitive liposomes (Philippot and Schuber, 1995).

Charged lipids

Charged lipids may be included in the membrane to increase the surface charge density to prevent close approach of liposomes and, hence, prevent aggregation, fusion, etc. Also, they are included in liposomal structure to push adjacent membranes apart within a multilamellar liposome to increase the internal aqueous volume. In negatively charged (acidic) phospholipids (e.g. cardiolipin, phosphatidylserine (PS), phosphatidylglycerol (PG), all three possible forces regulating headgroup interaction of bilayer membranes come into play, namely steric hindrance, hydrogen bonding, and electrostatic charge. No natural positively-charged phospholipids exist, however. Diacylglycerol, stearylamine, and dicetylphosphate have been incorporated into liposomes so as to impart either a negative or a positive surface charge to these structures. Generally about 10-20 mole percent of a charged species is used although it is possible to produce MLVs from a purely charged lipid such as PS.

LIPID	PHASE	MOLECULAR SHAPE
LYSOPHOSPHOLIPIDS DETERGENTS	MICELLAR	INVERTED CONE
PHOSPHATIDYLCHOLINE SPHINGOMYELIN PHOSPHATIDYLSERINE PHOPHATIDYLINOSITOL PHOSPHATIDYLGLYCEROL PHOSPHATIDIC ACID CARDIOLIPIN DIGASACTOSYLDIGLYCERIDE	BILAYER	CYLINDRICAL
PHOSPHATIDYLETHANOLAMINE (UNSATURATED) CARDIOLIPIN -Ca^{2+} PHOSPHATIDIC ACID -Ca^{2+} (pH<6.0) PHOSPHATIDIC ACID (pH<3.0) PHOSPHATIDYLSERINE (pH<4.0) MONOGALACTOSYLDIGLYCERIDE	HEXAGONAL (H_{11})	CONE

Figure 4. Effect of molecular geometry on phase properties of lipids (From Weiner, Martin, and Riaz, 1989).

Cholesterol

Cholesterol reduces the fluidity of membranes above the phase transition temperature (T_c), with a corresponding reduction in permeability to aqueous solutes. Consequently, inclusion of cholesterol into unsaturated membranes is often essential in order to achieve sufficient stability. On the other hand, cholesterol increases the fluidity of membranes below the phase transition temperature, so that its inclusion in saturated membranes, which are usually in the gel phase at ambient temperature, may result in a reduction in stability.

Liposomes without cholesterol tend to react with blood protein components such as albumin, m-transferrin, and macroglobulin. These components tend to destabilize the liposomes and reduce the utility of liposomes as drug delivery systems. Cholesterol improves stability of bilayer membranes in the presence of blood proteins.

Preparation of liposomes by reverse phase evaporation method
(Szoka and Papahadjopoulos, 1978)

Liposomes made by reverse phase evaporation (REV) method, developed by Szoka and Papahadjopoulos (1978), can be prepared by forming a water-in-oil emusion of phospholipids and buffer in excess organic phase followed by removal of the organic phase under reduced pressure (the so called "Reverse Phase Evaporation method). After the droplets have been formed by bath sonication of a mixture of the two phases, the emulsion is dried down to a semi-solid gel in a rotary evaporator, under reduced pressure. At this stage, the monolayers of phospholipid surrounding each water compartment are closely opposed to each other, and in some cases probably already form part of a bilayer membrane separating adjacent compartments (Figure 5). When vigorously shaken, the collapse of a certain proportion of the water droplets takes place. In these circumstances, the lipid monolayer which enclosed the collapsed vesicle is contributed to adjacent intact vesicles to form the outer leaflet of a large unilamellar liposome. The aqueous content of the collapsed droplet provides the medium required for suspension of these newly formed liposomes.

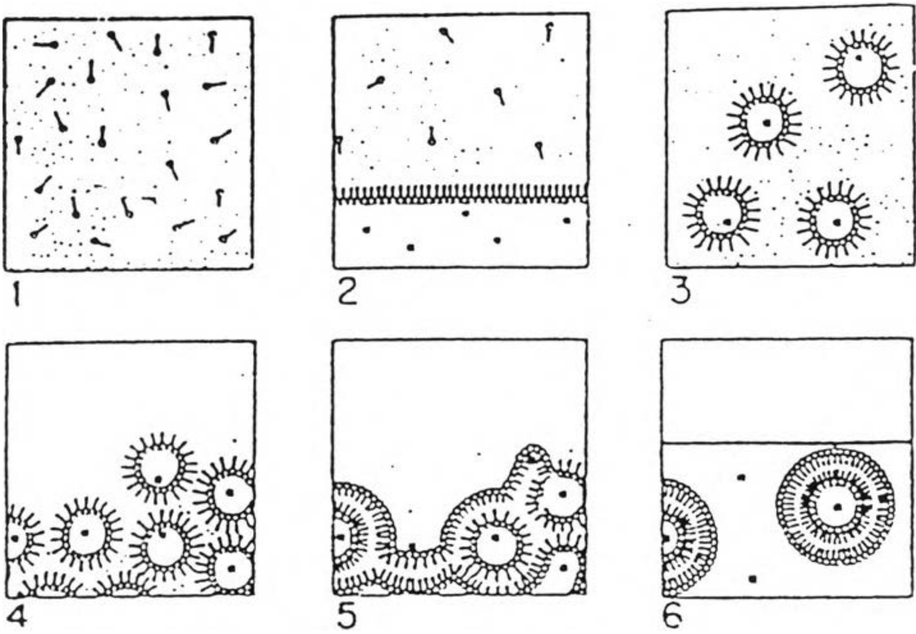

Figure 5. Diagram of the formation of REVs (From Szoka and Papahadjopoulos, 1978).

Drug entrapment in liposomes

(Weiner, Martin, and Riaz, 1989; Betageri, Jenkins, and Parsons, 1993; Sharma, 1997)

The amount and location of a drug within a liposome depends on a number of factors. The location of drug within a liposome is based on the partition coefficient of the drug between aqueous compartments and lipid bilayers. The maximum amount of drug that can be entrapped within a liposome, on the other hand, depends on its total solubility in each phase. The total amount of liposomal lipid used and the internal volume of the liposome will affect the total amount of non-polar and polar drugs that can be loaded into a liposome. Efficient capture will depend on the use of drugs at concentrations which do not exceed the saturation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for non-polar drugs). The method of preparation can also affect drug location and overall trapping efficiency. Figure 6 represents the various sites in the liposome available for drug entrapment.

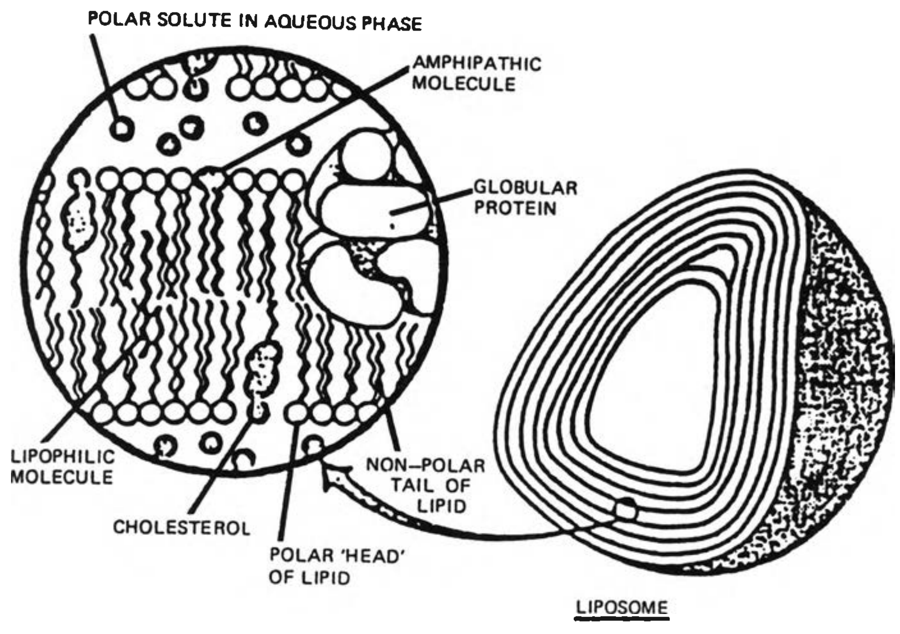

Figure 6. Diagrammatic representation the various sites in the liposome avaiable for drug entrapment (From Weiner, Martin, and Riaz, 1989).

Incorporation of drugs that have intermediate partition coefficients (significant solubility in both the aqueous phase and the bilayer) may be problematic because the drug molecules can diffuse in and out of the lipid membrane. For ionizable drugs, encapsulation efficiency may be improved by changing the pH of the aqueous medium. Any change in pH causes a change in the aqueous solubility of these drugs and thus affects their partitioning. For bilayer-associated drugs, the encapsulation depends on the mechanism of interaction. If the drug-liposome interaction is based on electrostatic forces, then encapsulation is affected by the density of the charge-inducing bilayer constituent and the ionic strength of the aqueous medium.

Parameters influencing liposomal characteristics

Liposomal formulations of various drugs can be optimized in terms of drug encapsulation, stability, the amount of drug released, and biological performance by altering their physicochemical parameters. The effects of these parameters on liposomal characteristics are as follows:

1. Type of vesicles

MLVs are suitable for the encapsulation of bilayer-interacting hydrophobic drugs and less appropriate for hydrophilic drugs because they have a low aqueous encapsulation volume. LUVs are more suitable for hydrophilic drugs due to their large entrapped aqueous volume, while SUVs have very low encapsulation efficiency for these drugs because of the low encapsulation volume (Stamp and Juliano, 1979; Szoka and Papahadjopoulos, 1980). Thus, for hydrophilic drugs, encapsulation appears to increase in the order of MLVs< SUVs< LUVs. However, entrapment of highly hydrophobic drugs is less affected by vesicle type, since they remain entrapped within the phospholipid bilayers.

2. Bilayer fluidity

Lipids have a characteristic phase transition temperature, and they exist in different physical states above and below the T_c. The lipids are in a rigid, well-ordered arrangement ("Solid" gel-like phase) below the T_c, and in a liquid-crystalline ("Fluid") phase above the T_c. The fluidity of liposomal bilayers can be altered by using phospholipids with different T_c which in turn can vary from –20 to 90 °C depending upon the length and nature (saturated or unsaturated) of the fatty acid chains. Presence of high T_c lipids (T_c >37 °C) makes the liposomal bilayer membrane less fluid at the physiological temperature and less leaky. In contrast, liposomes composed of low T_c lipids (T_c < 37 °C) are more susceptible to leakage of drugs encapsulated in aqueous phase at physiological temperatures. Incorporation of cholesterol into lipid bilayer can also affect the fluidity of the membranes. At high concentrations (>30 mole%), cholesterol can totally eliminate phase transition and decrease the membrane fluidity at a temperature > T_c, which makes the liposomes more stable and less leaky (Sharma, 1997).

3. Surface charge

Incorporation of charged lipids into bilayers increases the volume of the aqueous compartments by separating adjacent bilayers due to charge repulsion. This is particularly true in low ionic strength buffers or non-electrolytes since the electrostatic repulsive forces which give rise to the effect are greater under these conditions. The presence of charged lipids also reduces the likelihood of aggregation following the formation of MLVs (Weiner, Martin, and Riaz, 1989). Lack of surface charge can reduce physical stability of SUV by increasing their aggregation (Sharma and Straubinger, 1994). Neutral liposomes do not interact significantly with cells but high electrostatic surface charge could promote liposome-cell interaction (Sharma, 1997).

4. Method of preparation

Liposomes of different sizes and characteristics usually require different methods of preparation. The simple and widely used method for preparation of MLVs is the thin-film hydration method. However, one of the major disadvantages of this method is its relatively poor encapsulation efficiency (5-15%) of hydrophilic drugs. Moreover, reduction of liposomal size further decreases the amount of encapsulated drug (Sharma, 1997).

Several methods have been developed for the preparation of LUVs, including solvent (ether or ethanol) injection, detergent dialysis, and reverse-phase evaporation techniques. Using REV method, a maximum of 65% encapsulation may be obtained, but the major drawback of this method is that macromolecules such as proteins may get denatured by organic solvent (Szoka and Papahadjopoulos, 1978). SUVs can be prepared from MLVs or LUVs by sonication or extrusion.

Stability of liposomes

The stability of any pharmaceutical product is usually defined as the capacity of the formulation to remain within defined limits for a predetermined period of time (shelf-life of the product). The stability of liposomal products should preferably meet the standards of conventional pharmaceutical products. Both chemical and physical stability aspects are involved. Possible stability problems include loss of entrapped drug, change in liposome structure (including particle size distribution and aggregation/fusion of liposomes), and chemical instability of the liposomes and entrapped drug (Weiner, Martin, and Riaz, 1989; Cevc, 1993; Betageri, Jenkins, and Parsons, 1993).

1. Chemical stability

The chemical instability mainly concerns hydrolysis of the ester bonds in phospholipids and oxidation of their unsaturated acyl chains. These reaction products can cause dramatic changes in the permeability properties of liposomes. Preparative procedures or storage conditions can affect the decomposition rate of the liposomal lipids.

Lipid hydrolysis: The most important degradation product resulting from lecithin hydrolysis is lyso-lecithin (lyso-PC), which results from hydrolysis of the ester bond that link fatty acids to the glycerol moiety. The presence of lyso-PC in lipid bilayers greatly enhances the permeability of liposomes. It is therefore extremely important that the formation of lyso-PC be kept to a minimum during storage. The reaction become significant above 40 °C and are strongly pH-dependent with a pH of maximum stability around 6.5.

Lipid peroxidation: Most of the phospholipid liposomal dispersions used contain unsaturated acyl chains as part of the molecular structure. Double bonds present in these chains are vulnerable to oxidative degradation (lipid peroxidation). Phospholipids from common sources such as egg and soy are relatively inexpensive but, unfortunately, have a significant number of double bonds. Stability of these phospholipids can be achieved by

complete or partial hydrogenation, which is costly. Oxidation of the phospholipids may be minimized by the addition of an antioxidant (butylated hydroxytoluene, butylated hydroxyanisole, or α-tocopherol), protection of the phospholipids from light, storage under an inert gas such as argon or nitrogen to minimize exposure to oxygen, and the addition of EDTA to chelate any trace heavy metal ions. The other bilayer lipids which may be present can also decompose. For example, cholesterol, in aqueous dispersion, has been shown to oxidize rapidly when unprotected.

2. Physical stability

Two processes affecting the quality of liposomes have been focused for the physical stability of liposomes. First, the encapsulated drug can leak from the vesicles into the extra-liposomal compartment. Second, liposomes can aggregate and/or fuse, forming larger units. Both processes (leakage and aggregation/fusion) change the disposition of the compound in vivo and thereby can affect the therapeutic index of the compound involved. Two categories of approaches for stabilization can be discerned: the structure of the bilayer can be adjusted to induce optimum stability in the case of storage of aqueous liposomal dispersions and the aqueous liposomal dispersions can be freeze-dried (Cevc, 1993).

For all drugs, entrapment stability is better in MLVs than in SUVs. Stability is enhanced when the lipid portion of the phospholipids has long acyl chains, when liposomes are maintained at low temperature, and when phospholipids are in the gel state (Betageri, Jenkins, and Parsons, 1993).

Cholesterol is often added to leaky bilayers to reduce leakage rates (New, 1990). For hydrophilic, low molecular weight, and non-bilayer interacting compounds, selection of proper bilayer constituents is critical. For compounds that strongly interact with the bilayer, however, leakage is not a major problem.

Structure and function of the skin

The skin, by weight, is the largest organ of the body. Human skin serves to provide several important functions. These include maintaining physical protection (barrier function) against external agents and dehydration, receiving sensory stimuli from the environment, regulating body temperature and water balance, excreting a variety of substances, participating in metabolic pathways, and serving as a compartmentalized component of the immune system. As shown in figure 7, the skin is divided into three major layers: epidermis, dermis, and subcutaneous tissue (Lynch and Roberts, 1990; Schmitt, 1992; Roberts and Walters, 1998).

1. Epidermis

The epidermis consists of several layers and varies in surface topography and thickness, ranging from about 0.8 mm on the palms and the soles to 0.06 mm on the eyelids. The outermost layer of the epidermis is the stratum corneum. It consists of about 15 to 20 layers of flattened cells (keratinocytes), which become disassociated and ready for shedding or desquamation as they migrate to the surface. A total turnover of the stratum corneum occurs once every 2 to 3 weeks. The stratum corneum also functions as a barrier to prevent the loss of internal body components, particularly water, to the external environment.

The viable epidermis performs many functions. The generation of the stratum corneum starts as a germinative process in the germinative layer, the stratum basale, and undergoes differentiation into the stratum spinosum and stratum granulosum. Other functions include metabolism of substrates, including foreign substances, and the synthesis of melanin from melanocytes for skin pigmentation and sun protection. Other cells present in this layer are Langerhans cells (important in recognising the presence of antigens and facilitating an immune response), Merkel cells (with a role in sensory perception), and keratinocyte cell bodies (in contact with nerve fibers by membrane-membrane apposition).

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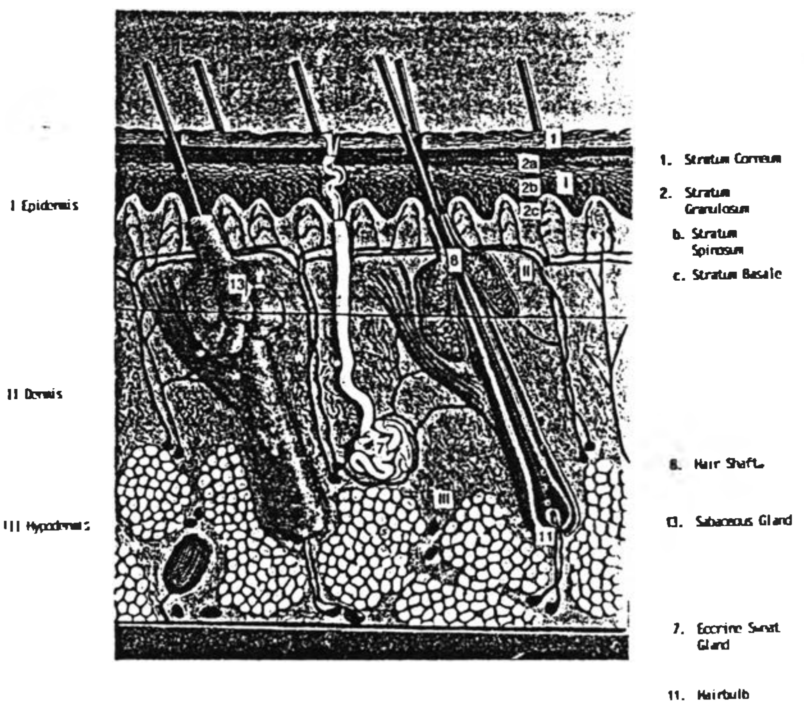

Figure 7. Cross-section of the skin showing epidermis (I); dermis (II); hypodermis (III); stratum corneum (1); stratum granulosum (2a); stratum spinosum (2b); stratum basale (2c); hair shaft (8); sebaceous gland (13); eccrine sweat gland (7); and hair bulb (11) (From Schmitt, 1992).

2. Dermis

The dermis not only provides the nutritive, immune, and other support systems for the epidermis through a thin papillary layer adjacent to the epidermis but also plays a role in temperature, pressure, and pain regulation. The main structural component of the dermis is referred to as a coarse reticular layer. The dermis is about 0.1 to 0.5 cm thick and consists of collagenous fibers, providing a scaffold of support and cushioning, and elastic connective tissue, providing elasticity, in a semigel matrix of mucopolysacchrides. In general, the dermis has a sparse cell population. The main cells present are the fibroblasts, which produce the connective tissue components of collagen, laminin,

fibronectin, and vitronectin; mast cells, which are involved in the immune and inflammatory responses; and melanocytes, which are responsible for the production of the melanin pigment.

Contained within the dermis is an extensive vascular network providing for the skin nutrition, repair and immune responses and, for the rest of the body, heat exchange, immune response, and thermal regulation. There are a number of different types of nerve fibers supplying the skin including those for pressure, pain, and temperature.

3. Subcutaneous tissue

The deepest layer of the skin is the subcutaneous tissue or hypodermis. It acts as a heat insulator and shock absorber as well as energy storage. This layer is a network of fat cells linked to the dermis by interconnecting collagen fibers.

Liposomes for dermal drug delivery

The topical route of administration has been utilized either to produce local effects for treating skin disorders or to produce systemic drug effects. The primary purpose of applying drugs to the skin is to induce local effects close to the site of application. In such cases, cutaneous absorption is required but percutaneous absorption is not. On the other hand, for systemic effect, the drug should penetrate through the skin and reach effective drug concentration at its site of action. In formulation of topical dosage forms, the use of various vehicles containing penetration promoters or drug carriers to ensure sufficient penetration and/or localization of the drug has been attempted to achieve local effects or to ensure sufficient percutaneous absorption to produce a desired systemic effect (Singh and Vyas, 1996).

The topical delivery of drugs by liposomal formulations has been widely of interest to increase drug absorption through the skin for both systemically and locally active drugs. Several groups have reported increased percutaneous absorption from

liposomal formulations leading to higher systemic levels for a given topical dose (Yu and Liao, 1996; Foldvari, 1994). Others have observed increased levels of drugs in the skin combined with decreased percutaneous absorption (Plessis, Weiner, and Muller, 1994; Mezei, 1990; Mezei and Getsztes, 1990). Still more have reported that liposomes can either reduce or increase cutaneous clearance, depending on the type and composition of the liposomal product (Imbert and Wickett, 1995). Some common mechanisms of (inter) action have been suggested (Schreier and Bouwstra, 1994). Most investigators agree that no transport of lipids takes place across whole skin (Ganesan.et al., 1984; Ho et al., 1985; Komatsu et al., 1986; Knepp et al.,1988; Knepp, Szoka and Gluy, 1990; Plessis, Egbaria and Weiner, 1992; Yu and Liao, 1996). Kirjavainen et al. (1996) showed that intact liposomes were not able to penetrate into the granular layers of epidermis. An exception is the hypothesis put forward by Cevc and Blume (1991), who maintains that so called transfersomes can transfer large amount of lipid in form of "lipid aggregates" to deeper layers of the skin. Most studies, however, are in agreement that direct contact between vesicles and skin is essential for penetration and drug delivery.

Although the mechanism of interaction of liposomes with skin is not clear, it does appear to be strongly dependent on liposomal parameters, such as the number of lamellar, surface charge, and particle size (Michel et al., 1992).

Liposomes can incorporate both lipophilic and hydrophilic drugs because they have both hydrophilic and hydrophobic compartments. They may serve as organic solvent for solubilization of poorly soluble drugs. They increase permeability of the skin for various entrapped drugs and, at the same time, diminish the side effects of these drugs (Plessis, Weiner, and Muller, 1994; Mezei, 1990; Mezei and Getsztes, 1990). They can be a local depot for the sustained release of dermally active compounds and enhance the accumulation of the drug at the site of administration (Schreier and Bouwstra, 1994). In addition, since the liposomal structure allows slow osmotic diffusion of water or water soluble agents through the lipid membrane onto the skin (Idson, 1995), a liposomal formulation of drug should retain skin moisture and decrease skin irritation. Lipid components of liposomes which are arranged in bilayers can also prevent water evaporation from the skin (Idson, 1985).

Liposome - Cell interactions

(Poste and Papahadjopoulos, 1976; Betageri, Jenkins and Parsons, 1993; Gregoriadis, 1993; Miller, 1998; Duzgunes and Nir, 1999)

Liposomes have long been considered good candidates for efficient drug carrier and delivery systems. They offer a local delivery of therapeutic agents to the site of interest while reducing systemic toxicity. If they are able to reach a specific site they may release their contents in the vicinity of the cells and/or be taken up by the cells. Liposomes can interact with cells in several ways to cause liposomal components to become associated with those cells.

1. Intermembrane transfer

Intermembrane transfer of lipid components can occur upon close approach of the phospholipid bilayers without disruption of liposomal integrity. Such transfer may occur with complete retention of the components of the liposomal aqueous compartment.

Exchange of liposomal phospholipids with cells may occur through a specific cell surface exchange protein since only certain phospholipids exchange (PC and PE). In addition, the process is slowed after trypsin treatment. The mechanism of lipid or protein transfer from cell membrane to liposomes is not clear. Phospholipid transfer may occur when liposomes collide with the cell surface. Special lipid transfer proteins on the cell surface could mediate this process.

2. Contact release

Liposome contact with the cell causes an increase in permeability of the liposomal membrane. This leads to release of water-soluble solutes in high concentration in the close vicinity of the cell membrane through which these solutes may, under certain circumstances, then pass. The cell-induced leakage of solutes is greater in membranes

with chotesterol concentrations above 30 mole%. This leakage is attributed to the interaction of cell surface proteins with the liposomal bilayers.

The above two phenomena can provide very effective means for introducing materials into specific cells without ingestion of liposomes. These phenomena would be of particular value for cells which are not actively phagocytic. These methods will be most effective under conditions where flow and turbulence of the medium surrounding the cells are reduced and where physical interactions between liposomes and cells are strengthened through receptor/ligand binding. Whether these processes take place to a significant extent depends upon membrane composition and the nature of the compounds themselves.

3. Adsorption

Adsorption of liposomes to the cell surface can often occur with little or no internalization of either aqueous or lipid components. Adsorption may be due to physical attractive forces or binding by specific receptors to ligands on the liposomal membrane. Physical adsorption of liposomes may occur through binding to a specific cell surface protein. Uptake is greatest at or below the phase transition temperature of the liposomal membrane, presumably because the binding sites involved are membrane defects which are more stable at low temperature. Adsorption is an essential prerequisite for liposome ingestion by cells. Attachment of liposomes to cell membranes via certain surface proteins results in rapid uptake into the cell.

4. Fusion

Close approach of liposomes and cell membrane can also lead to fusion. Fusion can result in complete mixing of liposomal and cell plasma membrane lipids and release of liposomal contents into the cytoplasm. Fusion was once considered the dominant mechanism of cell interaction with liposomes in a fluid state. However, spontaneous fusion between liposomes and cell membrane is rare. This is because the cell membrane preserves independence of the cell from other cells and from environmental factors.

occurs readily by incorporation of fusogens (e.g. lysolecithin, PS, detergents and surfactants) into the membrane. However, these materials may be quite toxic to cells presumably through nonspecific membrane perturbing effects after incorporation within the cells.

5. Phagocytosis / Endocytosis

Cells with phagocytic activity take liposomes into endosomes (subcellular vacuoles formed by invagination of the plasma membrane), which have a pH of 5 to 5.5. Endosomes then fuse with lysosomes to form secondary lysosomes where cellular digestion occurs in a milieu of approximately pH 4.5. Lysosomal enzymes break open the liposomes, hydrolyzing the phospholipids to fatty acids, which can be recycled and reincorporated into host phospholipids. During liposomal breakdown in lysosomes, the contents of the aqueous compartment are released. These contents will either remain sequestered in the lysosomes until exocytosis (particularly if they are highly charged at low pH), or they will slowly leak out of the lysosome into the cell.

In addition to phagocytosis, liposomes may also be taken up by receptor-mediated endocytosis. Liposomes coated with low density lipoproteins (LDL), or transferrin, bind to the cells via surface receptors and are internalized via coated pits, with subsequent ligand degradation or recycling. Thereafter, liposomes may gain access to subcellular compartments (e.g., Golgi) other than lysosomes.

Factors influencing the uptake of liposomes into cells

Composition

The nature and density of charge on liposomal surface are important parameters influencing the mechanism and extent of liposome-cell interaction. Both of these parameters can be altered by changing the lipid compositions. Neutral liposomes do not interact significantly with cells, and in such cases, the drug may mainly enter cells after

interact significantly with cells, and in such cases, the drug may mainly enter cells after being released from liposomes extracellularly. On the other hand, high electrostatic surface charge could promote liposome-cell interaction. It has been shown that negatively charged liposomes are predominantly taken up by cells through coated-pit endocytosis. Negative charge density also influences the extent of interaction of liposomes with cells (Sharma, 1997).

Bajoria, Sooranna, and Contractor (1997) suggested that the uptake of liposomes depended upon their surface charge. The carboxyfluorescein-encapsulated anionic liposomes (PC/CH/DCP, 7: 7:1) were taken up by the trophoblast cells more avidly than the neutral (PC/CH, 1:1), the cationic (PC/CH/SA, 5:5:0.05), and the free carboxyfluorescein.

Liposomes containing anionic phospholipids could undergo membrane fusion in the presence of calcium ions. These observations by Papahadjopoulos and co-workers are particularly relevant to the fusion of biological membranes, since Ca^{2+} is involved in the control of exocytosis, myoblast fusion, and other biological membrane fusion phenomena.

Papahadjopoulos et al. (1973) found that liposomes of certain phospholipid composition could induce the fusion of cultured cells. Liposomes composed of pure PS or its 1:9 mixture with PC caused extensive cell-cell fusion in cultures of 3T3, L929, and BHK21 cells, while pure PC liposomes did not induce any fusion.

Cationic liposomes have been proposed to deliver contents to cells by fusion with cell membranes (Sharma, 1997). Alpar et al. (1990) revealed that positively charged liposomes composed of PC/CH/SA (7:2:1) were most effective in the delivery of fluorescein isothiocyanate-albumin to HL-60 cells, a non-phagocytic leukaemic human cell line, while those with a negative charge (PA) showed very little transference ability.

Magee et al. (1974) studied liposome-cell interactions utilized liposomes containing the positively charged lipid stearylamine. The liposomes adhered to HeLa

cells and were endocytosed. Membrane fusion was proposed to account for the transfer of some of the encapsulated enzyme to the cell membrane. Another study reported that liposomes composed of PC, lyso-PC, and stearylamine could mediate cell-cell fusion (Martin and MacDonald, 1976).

The analysis of the results of Ca^{2+}-induced fusion of large unilamellar PS/CH vesicles showed that cholesterol promoted the overall rate of vesicle fusion. (Philippot and Schuber, 1995). Hsu et al. (1983) showed a cholesterol requirement in fusion, an optimum being observed at 0.3-0.4 mole fraction of cholesterol. In contrast, no effect of cholesterol was observed by Haywood and Boyer (1984).

Bilayer fluidity

The phase state of the liposomes is also an important factor in the liposome-cell interaction. Negatively charged solid vesicles (PS/DPPC/DSPC, 1:4.5:4.5) are incorporated into BALB/c mouse 3T3 cells primarily by endocytosis. In contrast, the major in corporation pathway for fluid charged vesicles (PS/PC, 1:9) is via a non-endocytotic mechanism, which Poste and Papahadjopoulos (1976) proposing fusion of lipid vesicles with the plasma membrane. Liquid crystalline state PG/PC (1:9) liposomes or DPPG/DPPC (1:9) liposomes in an intermediate phase induce greater fusion than DSPG/DSPC (1:9) liposomes in the gel phase (Papahadjopoulos, Poste and Mayhew, 1974).

Size

Uptake of liposomes will depend on physical parameters such as their size relative to the size of endocytic vesicles of target cells. Liposomes cannot be endocytosed into compartments which are smaller than themselves (Gregoriadis, 1993). The optimal size for endocytosis of LUVs is 0.05-0.1 μm. Liposomes larger than 0.4 μm cannot deliver entrapped substances into the cytoplasm (Betageri, Jenkins, and Parsons, 1993).

The curvature of liposomes might facilitate fusion, affecting thereby the relevance of the results to biological membrane fusion, particularly with small vesicles (Philippot and Schuber, 1995). Monkkonen et al. (1994) reported that a decrease in the size of unilamellar DSPG liposomes by extrusion did not affect the delivery of the compounds to RAW 264 macrophages, but drastically increased their potency for L929 fibroblasts.

Cells

Liposome uptake by different cells has a varying dependence on the liposomal composition. The presence of a relatively small mole percentage of certain negatively charged phospholipids (PS, PG, or PA) in the liposomal membrane enhanced uptake by CV1 cells, while uptake by J774 macrophages was not affected (Straubinger et al., 1983). The liposomal membrane had to contain at least 50 mole% PS, PG, or PI in order to achieve the same level of uptake in J774 cells as in CV1 cells. These experiments suggested that different cells have different liposome-binding sites on their surface. Neutral PC liposomes bind to V79 fibroblasts via trypsin-sensitive sites, while the transfer of carboxyfluorescein from dioleoyl-PC liposomes to primary lymphocytes is not affected by trypsin or pronase (Pagano and Takeichi, 1977; Blumenthal et al., 1977).

Liposomal Toxicity

(Betageri, Jenkins and Parsons, 1993; Gregoriadis, 1993; Stanzl, 1999)

The potential toxicity of liposomes depends upon the type of lipids used. The phospholipids usually employed in liposomes are biodegradable and similar to those found in commercially available preparations for parenteral nutrition, all of which are essentially nontoxic. However, direct evidence of toxic effects due to the presence of certain lipids in the bilayers has been accumulated in vitro. From most in vitro experiments, charged lipids have been implicated in the toxicity of liposomes. Many animal studies on acute toxicity of liposomes also indicate that, as with the in vitro situation, liposomal lipid components with a net charge are primarily responsible for

acute toxicity. Neutral liposomes composed of PC and CH appear to be tolerated better than charged liposomes.

Most reports concerning the administration of SA-containing liposomes in vitro and via several routes (i.v., s.c., i.c.) suggest that SA is potentially toxic (Gregoriadis, 1993). Binding of the SA-liposomes to negative charges on cell surface might be involved. Alternatively, rapid release of SA from the liposomes followed by incorporation in various plasma membranes may mediate pharmacological effects. With respect to the use of negatively charged lipids, available literature suggests that PA and PG are safer alternatives to DCP and PS.

However, various variations in each specific experimental design such as physical and chemical properties of the formulation, the cell lines used, the dose, and the method of application should also be considered when evaluation of liposomes is undertaken in vitro. Such variations have made it difficult to compare results from different laboratories.

Cell culture

(Freshney, 1994)

Cell culture is a method for studying the behavior of animal cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of an experiment. Cell cultures may be derived from primary explants, a fragment of tissue is placed at a glass (or plastic) - liquid interface where, following attachment, migration is promoted in the plane of the solid substrate, or dispersed cell suspensions. Because cell proliferation is often found in such cultures, propagation of cell lines becomes feasible. A monolayer or cell suspension, with a significant growth fraction, may be dispersed by enzymatic treatment or simple dilution and reseeded, or subcultured, into fresh vessels. This constitutes a passage and the daughter cultures so formed are the beginnings of a cell line. The formation of a cell line from a primary culture implies (1) an increase in total cell number over several generations and (2) that cells or cell lineages

with similar high growth capacity will predominate, resulting in (3) a degree of uniformity in the cell population. The line may be characterized, and those characteristics will apply for most of its finite life-span. The derivation of continuous cell lines usually implies a phenotypic change or transformation. When cells are selected from a culture, by cloning or by some other method, the subline is known as a cell strain. Detailed characterization is then implied. Cell lines, or cell strains, may be propagated as an adherent monolayer or in suspension.

Advantages of cell culture

The three major advantages of cell culture are the control of the environment, the characterization and homogeneity of sample, and the economy.

In control of the environment, both physiochemical environment (pH, temperature, osmotic pressure, O_2, and CO_2 tension) and physiological conditions are controlled. However, most cell lines still require supplementation of the medium with serum or other poorly define constituents. These supplements are prone to batch variation and contain undefined elements such as hormones and other regulatory substances.

Tissue samples are invariably heterogeneous. Replicates even from one tissue vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture each replicate sample will be identical, and the characteristics of the line may be perpetuated over several generations or indefinitely if the cell line is stored in liquid N_2. Since experimental replicates are virtually identical, the need for statistical analysis of variance is reduced.

Cultures may be exposed directly to a reagent at a lower and defined concentration, and with direct access to the cell. Consequently, less amount is required than for injection in vivo where 90 % is lost by excretion and distribution to tissues other than those under study. Screening tests with many variables and replicates are cheaper, and the legal, moral, and ethical questions of animal experimantation are avoided.

Disadvantages of cell culture

Culture techniques must be carried out under strict aseptic conditions. This requires a level of skill and understanding to appreciate the requirements of the system and to diagnose problems as they arise. In addition, the expenditure of effort and materials that goes into the production of relatively little tissue is a limitation of cell culture.

In cell line propagation, many workers observed the loss of the phenotypic characteristics typical of the tissue from which the cells had been isolated. Under the correct culture conditions, however, many of the differentiated properties of these cells may be restored. If differentiated properties are lost, it is difficult to relate the cultured cells to functional cells in the tissue. Stable markers are required for characterization, in addition, the culture conditions may need to be modified so that these markers are expressed.

Instability is a major problem with many continuous cell lines, resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures of untransformed cells, heterogeneity in growth rate and capacity to differentiate within the population can produce variability from one passage to the next.

Although the existence of differences between the environmental conditions of a cell in vitro and in vivo can not be denied, it must be emphasized that many specialized functions are expressed in culture, and as long as the limits of the model are appreciated, it can become a very valuable tool.