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Recent advances in delivery systems for anti-HIV1 therapy

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Abstract

In the last years, different non-biological and biological carrier systems have been developed for anti-HIV1 therapy. Liposomes are excellent potential anti-HIV1 carriers that have been tested with drugs, antisense oligonucleotides, ribozymes and therapeutic genes. Nanoparticles and low-density lipoproteins (LDLs) are cell-specific transporters of drugs against macrophage-specific infections such as HIV1. Through a process of protein transduction, cell-permeable peptides of natural origin or designed artificially allow the delivery of drugs and genetic material inside the cell. Erythrocyte ghosts and bacterial ghosts are a promising delivery system for therapeutic peptides and HIV vaccines. Of interest are the advances made in the field of HIV gene therapy by the use of autologous haematopoietic stem cells and viral vectors for HIV vaccines. Although important milestones have been reached in the development of carrier systems for the treatment of HIV, especially in the field of gene therapy, further clinical trials are required so that the efficiency and safety of these new systems can be guaranteed in HIV patients.

Keywords: *HIV1 therapy, carrier systems, drug delivery, gene therapy*

Introduction

In recent years, the struggle against HIV/AIDS has enhanced the development of different therapeutic strategies, involving two approaches: research and development of new anti-HIV drugs and gene therapy through the use of DNA vaccines.

Anti-HIV drugs, often known as antiretroviral drugs, are the most frequent alternative to establish control over the reproduction of the virus and to guarantee slow progression of the disease. Antiretroviral drugs are classified in four main groups: non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs) and fusion inhibitors. These drugs are usually used in combined therapy, such as in the case of highly active antiretroviral therapy (HAART). HAART therapies still have important limitations such as the high cost of the treatments, the adverse effects of some antiretroviral drugs, drug resistance, drug interactions, non-compliance problems, etc. (Kalkut 2005).

Gene therapy has been proposed as an alternative for human immunodeficiency disease (HIV1) (Luque et al. 2005; Dropulic and June 2006; Von Laer et al. 2006). Gene therapy for HIV1 infection is based on two main strategies. The first uses lethal genes to kill the infected cells before the virus can produce infective particles, while the second strategy involves protecting the cell from the virus (Luque et al. 2005).

RNA-based strategies for anti-HIV therapy based in the use of antisense oligonucleotides, small interfering RNA, RNA decoys and ribozymes are currently in progress, including combinatorial gene therapy strategies (Akkina et al. 2003; Nielsen et al. 2005; Ramezani et al. 2006).

Antisense oligonucleotides are short sequences of nucleic acids complementary to a given messenger nucleic acid (sense sequence). Antisense oligonucleotide therapy permits the inhibition of gene expression in HIV infection. The problems associated with this type of therapy essentially derive from the short half-life of antisense oligonucleotides and the difficulty

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involved in accessing the inside of the cell. Many modifications have been made for oligonucleotide which can significantly improve the half-life, stability and cellular uptake. Toxic effects such as mild thrombocytopenia and hyperglycemia, complement activation and coagulation cascades and hypotension are all associated with the use of antisense therapy (Zelphati et al. 1994; Putnam 1996; Jason et al. 2004; Phillips 2005).

Small interfering RNA or silencing RNA (siRNA) are a class of 20–25 nucleotide-long RNA molecules that interferes with the expression of a specific gene. siRNA may protect stem cells from HIV infection (Lamothe and Joshi 2000; Akkina et al. 2003; Song et al. 2003). RNA decoys inhibits the capacity of HIV1 Tat protein for the transcription of HIV1 genome (Bohjanen et al. 1997). Ribozymes are RNA molecules with catalytic activity. Combinatorial use of rybozymes in cultured cells have recently demonstrated the supression of HIV1 replication (Ikeda et al. 2006).

HIV/AIDS vaccines are an interesting alternative under investigation since currently no vaccine has been approved for routine clinical use. Basically, there are two types of HIV vaccines: the preventive type, whose main objective is to prevent HIV infection and therapeutic vaccines, which aim to improve the immune system in HIV-positive patients. Outstanding in this field are the canarypox vaccines or the DNA vaccines, among others (Dorrell 2005; Lambert 2005; Tubiana et al. 2005). Drugs with potent anti-HIV1 activity *in vitro* are ineffective or have a limited activity *in vivo* due to limited uptake in target cells. Different biological and non-biological delivery systems have been developed in order to solve this problem. In recent years, non-biological carrier systems, such as liposomes, nanoparticles, LDL or peptides and biological carriers, such as viral vectors, erythrocyte ghosts, stem cells or bacterial ghosts that permit specific *in vivo* intracellular delivery of anti-HIV1 drugs, have been developed with a view to improving their cellular uptake and enhancing their activity. Delivery systems allow the therapeutic agent to be vectors to organs of the reticuloendothelial system (RES), where the virus often resides and multiplies. In this way, it is possible to reduce the doses administered, improving efficiency and decreasing toxicity. The ability of such carriers to vehicle drugs into cells is increased by the fact that HIV-infected macrophages show greater phagocytic behaviour than those that are not infected (Löbenberg et al. 1998). Delivery systems can also be used in the field of gene therapy, especially with antisense oligonucleotides (Figure 1) and with anti-HIV vaccines (Cockrel and Kafri 2003).

Table I shows the non-biological and biological delivery systems for drugs and genetic material developed for anti-HIV1/AIDS therapy.

The aim of this review is to discuss recent advances in non-biological and biological delivery systems used

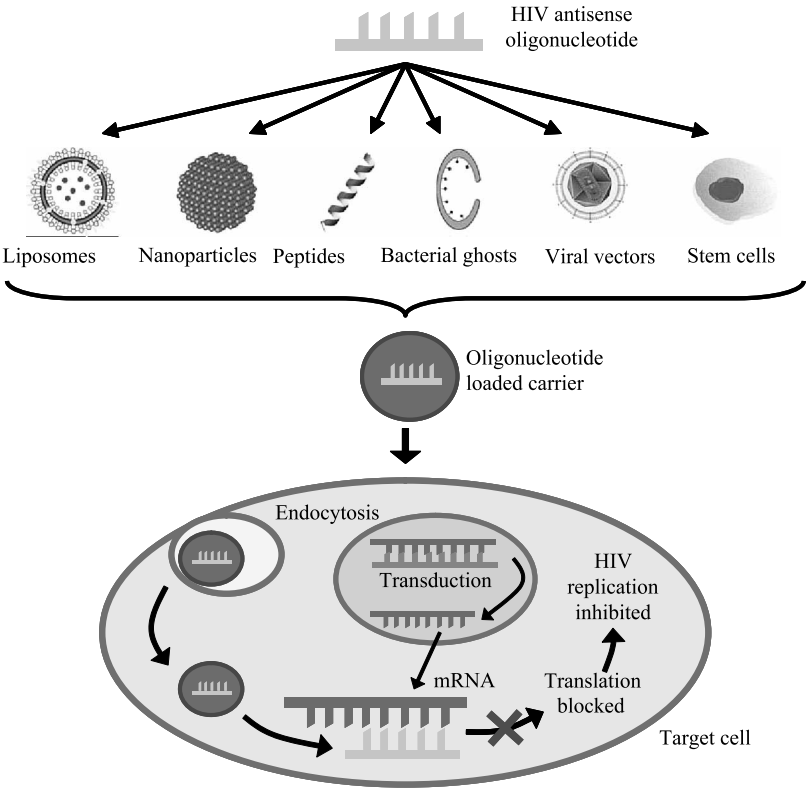

Figure 1. Carrier systems for antisense oligonucleotide therapy.

Table I. Classification of carrier systems used for anti-HIV1 therapy.

Carrier		Encapsulated drug	Reference
Non-biological carrier systems	Liposomes		
	Anionic liposomes	Stavudine (d4T)	Katragadda et al. (2000)
		ddCTP	Oussoren et al. (1999)
		Zidovudine	Jin et al. (2005)
	Cationic liposomes	Anti-HIV Rev-binding aptamer, ribozymes	Konopka et al. (1998a,b)
		Ribozymes	Kitijima et al. (1997)
	Immunoliposomes	Rev antisense phosphorothioate oligonucleotide	Selvam et al. (1996)
		Rev antisense phosphodiester or phosphorothioate oligonucleotide	Zelphati et al. (1993,1994)
		Env region antisense RNA	Renneisen et al. (1990)
		Indinavir	Gagne et al. (2002), Desormeaux and Bergeron (2005)
	Sterically stabilized liposomes	Indinavir	Gagne et al. (2002), Desormeaux and Bergeron (2005)
	Nanoparticles	Zalcitabine, saquinavir	Bender et al. (1996)
		Zidovudine	Löbenberg et al. (1997, 1998)
		DNA vaccines	Cui and Mumper (2003)
	LDL	5′-*O*-13-oxamyristate-AZT	Hu et al. (2000)
		Flucytosine, AZT	Mankertz et al. (1996)
	Peptides	Oligonucleotides	Lochmann et al. (2004)
		AZT	Fridkin et al. (2005), Giammona et al. (1998, 1999)
		Ganciclovir, acyclovir	Cavarallo et al. (2004)
Biological carrier systems	Erythrocytes	ddCTP	Magnani et al. (1992, 1995)
		AZTp2AZT	Benatti et al. (1996)
		GSH	Fraternale et al. (2001, 2002, 2003)
		PMEA	Perno et al. (1997)
		Bis-PMEA	Rossi et al. (2001)
		ACVpPMPA	Franchetti et al. (2000)
	Viral vectors		
	Poxviruses	Recombinant HIV1 antigens vaccines	Slyker et al. (2005)
		Canarypox ALVAC-HIV vaccines	Goepfert et al. (2005)
		Recombinant fowlpox virus vaccines	Coupar et al. (2006)
	Adenoviruses	Plasmid DNA vaccines	Xin et al. (2005) and Casimiro et al. (2003)
		rAAV/SIV vaccines	Johnson et al. (2005)
	Retroviruses	Gag-pol, env genes	Marusich et al. (2005)
	Stem cells	Anti-HIV ribozyme	Amado et al. (1999)
		Transdominant mutant Rev protein	Mautino et al. (2001) and Su et al. (1997)
		RRE-anti-HIV1 gene	Kohn et al. (1999)
		REVNL3-huM10, REVNL3-Fx genes	Podsakoff et al. (2005)
	Bacterial ghosts	DNA vaccines	Ebensen et al. (2004), Walcher et al. (2004) and Szostak et al. (1993)

with drugs and genetic material in anti-HIV therapy, their advantages, limitations and potential clinical applications.

Cellular targets for anti-HIV1 therapy

CD4+ cells are the primary targets for HIV infection, but HIV infects and replicates in many other cell types in the body, including other T cells or macrophages. Other cells, like dendritic cells, CD8+ or B lymphocytes and neutrophils have been found to bind and transfer infectious virus to these target cells (Levy 2002).

HIV infection starts with adsorption of a HIV virion to the cell membrane, through specific interaction with the cell surface receptor CD4 that is expressed mainly in T cells and monocyte-macrophages. It constitutes an interesting cellular target and several CD4 mimic molecules as well a small molecular weight drug named BMS-378806 have been developed to inhibit infection of the cell (Reeves and Piefer 2005).

However, HIV is also able to infect CD4− cells. The mechanism is not yet known, although there have been different hypothesis. Some authors have proposed that the presence of a membrane protein, claudin-7 on the target cells increase viral susceptibility and may have an important role in the infection of this type of cells (Zheng et al. 2005).

The chemokine receptors CCR5 or CXCR4 are the major coreceptors for HIV1 entry. CCR5 is an attractive target since it was described that human subject carrying a 32 base pair deletion in CCR5 are resistant to HIV infection (Huang et al. 1996; Liu et al. 1996; Samson et al. 1996). Some molecules that inhibit these co-receptors are being developed, like the current leading CCR5 antagonists vicriviroc, maravirok and aplavirok (Dorr et al. 2005; Reeves and Piefer 2005; Strizki et al. 2005). Also, several peptides and small molecular compounds that are inhibitors of the CXCR4 co-receptor have been assayed (Altmeyer 2004).

Among several other cellular proteins, cyclin-dependent kinases (cdks) are required for replication of HIV1. Pharmacological cdk inhibitors have proven to have potent *in vitro* activity against HIV1, like flavopiridol and roscovitine. However, other cellular proteins that are known to be required for viral replication may be good targets for antiviral drugs (Gibbs and Sorensen 2000; Zala et al. 2000; Tam et al. 2001), like cyclophilin (CYPA) a isomerase that is specifically incorporated into newly formed HIV1 virions (Sokolskaja and Luban 2006). Those cellular proteins showing upregulation of expression during viral infection should be analysed as potential targets for antiviral drugs (Fruh et al. 2001).

The host proteome offers also numerous potential therapeutic targets. To identify cellular proteins that may play a role in infection, the effect on the trans-activating transcriptional activator (Tat) on cellular gene expression was analysed and a list of gene products that may serve as potential therapeutic targets for the inhibition of viral replication was compiled, including PouAFI (OBF-1), complement factor H related 3, CD4 receptor, ICAM-1. NA and Cyclin AI (Liang et al. 2005; Shang 2002).

Also some intrinsic antiretroviral factors that restrict HIV infections have been identified as the TRIM 5α in mouse cells and Ref1 antiretroviral resistance factor in humans. It remains to be determined if variants of this proteins may have therapeutic utility for HIV infection (Hatziioannou et al. 2004; Keckesova et al. 2004; Yap et al. 2004).

Non-biological delivery systems

Conventional drug delivery systems such as liposomes and nanoparticles, among others, play an important role in drug therapy and especially in the field of cancer therapy (Ranade and Hollinger 2004). Among other advantages, this type of system allows the efficiency of the active ingredient to be increased; it reduces toxicity and modifies the pharmacokinetic behaviour of the drug and in particular, it facilitates selective access of the drug to its site of action, which is sometimes intracellular.

Non-biological carrier systems such as liposomes, nanoparticles, LDL and different types of peptides have been assayed as potential carriers of drugs and genetic material for anti-HIV therapy.

Liposomes

Liposomes are a classic non-biological delivery system used with different drugs and have many therapeutic applications. Liposomes are defined as microscopic vesicles formed by one or several phospholipid bilayers surrounding an aqueous compartment, with a particle size between 25 nm and several microns, such that they are able to encapsulate hydrophilic drugs inside the aqueous phase, or hydrophobic drugs bound to or incorporated in the lipid bilayer. They are usually made up of phospholipids, natural or synthetic and cholesterol and they may incorporate other lipids and derivatives and also proteins. Among the advantages of liposomes is the fact that they are biodegradable, sparingly toxic and immunogenic and they show a high drug/vehicle ratio (Agrawal and Gupta 2000; Kozubek et al. 2000). However, they also have some disadvantages, such as their poor stability, both in the bloodstream, due to the presence of serum lipoproteins and in storage; there is also the problem of the low encapsulation efficiency of the methods used to elaborate them, the presence of solvent residues in the final preparation, which is unacceptable owing to their possible toxicity and the high costs of industrial production (Prior et al. 2002).

When liposomes are administered *in vivo*, they are rapidly eliminated from the circulation by monocytes and macrophages and are accumulated in cells of the RES, especially the liver and spleen. This characteristic of liposomes is an advantage in anti-HIV therapy because macrophages are an important reservoir of HIV.

The existence of negative charges favours the binding of serum proteins to the lipid surface, in turn favouring phagocytosis and increasing the resistance to serum HDL and hence providing stability in the blood stream. Also, cationic liposomes show greater monocyte activation. The electrostatic attraction between the positive charges of the liposome and the negative charges of the cellular surface may also favour certain liposome internalization mechanisms, such as fusion or endocytosis (Vitas et al. 1996).

Cholesterol or sphingomyelin are often included in the composition of liposomes in order to increase the rigidity of the lipid bilayer, which hinders interactions with the cell. Accordingly, the presence of increasing amounts of cholesterol will decrease the cellular penetration of the vesicles (Katragadda et al. 2000; Ahsan et al. 2002). Cholesterol also favours liposome stability against serum lipoproteins and the increase in the time of circulation in the blood and it decreases the percentage of encapsulation of the drug because it modifies the thickness of the membrane (Karlowsky and Zhanel 1992; Vitas et al. 1996).

There are essentially four types of liposomes: anionic liposomes, cationic liposomes, immunoliposomes and sterically stabilised liposomes. All these types are candidates for use as carriers of drugs and genetic material in anti-HIV therapy.

To be pharmacologically active, antiretroviral drugs that are inhibitors of transcriptase must be phosphorylated to 5′ triphosphate by cellular kinases. Owing to the poor ability of some cells to phosphorylate these compounds, the alternative would be the administration of the phosphorylated form directly. However, this is not possible because of the low permeability of cellular membranes to nucleotides and their rapid hydrolysis in biological fluids (Oussoren et al. 1999). The inclusion of these drugs in liposomes has proved to be a good alternative in the treatment of AIDS. Stavudine (d4T) is a transcriptase inhibitor. Its incorporation in liposomes with different compositions in all cases elicited an increase in cellular penetration in human macrophages (Katragadda et al. 2000). In infected mice, the triphosphorylated form of dideoxycytidine (ddCTP) incorporated in liposomes reduces the content of proviral DNA in spleen and bone marrow. Signs of the disease, such as hypergammaglobulinaemia, lymphadenopathy and spleenomegaly decreases, with no modification to any blood parameters (Oussoren et al. 1999). The incorporation onto the surface of liposomes of molecules such as soluble CD4 and CD4-IgG, which inhibit HIV infectivity, decreases the selectivity to infected macrophages (Raulin 2002).

Zidovudine (AZT) is a very useful drug in antiretroviral therapy but it has several secondary effects. AZT shows dose-dependent toxic effects, especially in bone marrow. At high concentrations, it may also cause anaemia and leucopenia. Another problem with AZT is its short half-life, which means that administrations must be frequent in order to maintain its plasma levels within the therapeutic range. The use of AZT drug delivery systems allows sustained plasma levels to be maintained.

Recent pharmacokinetic studies carried out in the rat with zidovudin myristate-loaded liposomes administered intravenously have shown a sustained release effect characterised by an increase in drug plasma levels and in the area under the curve of AZT when administered in liposomes in comparison with administration in aqueous solution. At the same time, the administration of AZT incorporated into liposomes allowed a better accumulation of the drug in the RES (Jin et al. 2005).

Cationic liposomes as carriers are safe and non-immunogenic for *in vivo* gene delivery. Cationic liposomes are characterised by the fact that the cationic components of the outside interact with the negatively charged molecules of DNA, producing a system able to carry genetic material. In this type of liposome, the positive charge of the lipid allows interaction with the cell surface, permitting the selective release of genetic material to the target cell, reaching the nucleus and allowing expression of the therapeutic gene.

Cationic liposomes containing antisense oligonucleotides and ribozymes facilitate cytoplasmic delivery to target cells for the inhibition of gene expression and protect anti-HIV1 drugs from nuclease digestion. The cellular uptake of ribozymes complexed with cationic liposomes was higher than naked ribozymes and ribozymes complexed with anionic liposomes, although this type of carrier of genetic material has some problems *in vitro*, since the antiviral effect of the liposome-complexed ribozyme was not sequence-specific, limiting its potential applications *in vivo* (Kitajima et al. 1997; Konopka et al. 1998a,b; Duzgunes et al. 2001). Recent studies *in vitro* using a T cell line PBMC's infected with HIV1 and *in vivo* using mouses carried out with cationic liposomes containing phosphorothioate oligonucleotides demonstrate a enhanced cellular uptake both *in vitro* and *in vivo* expressed as the inhibition of the production of p24 antigen (Miyano-Kurosaki et al. 2004).

Immunoliposomes are characterised by having on their surface specific antibodies or fragments of antibodies and in this way they promote a specific action. Many studies *in vitro* and *in vivo* in experimental animals have demonstrated the inhibition of the expression of HIV1 using antibody-targeted liposomes containing antisense oligonucleotides or anti-HIV1

drugs (Zelphati et al. 1993; Selvam et al. 1996; Bestman-Smith et al. 2000; Gagne et al. 2002; Desormeaux and Bergeron 2005). Preliminary *in vitro* studies using antisense RNA encapsulated in protein A-bearing liposomes inhibited HIV expression. Protein A-liposomes were directed to target cells using anti-CD3 monoclonal antibodies. This study demonstrated that a dose-dependent inhibition of HIV1 replication in anti-CD3− treated cells could be achieved using a env-coding RNA in the antisense orientation while the same RNA sequence in the sense orientation was not effective (Renneisen et al. 1990). Later studies carried out *in vitro* with liposomes containing antisense nucleotides revealed that this type of liposome may inhibit viral replication in acutely and chronically HIV1-infected cells in tissue culture. In acute infection, the increase in the anti-HIV effect is related to the delivery of the antisense oligonucleotide carried in liposomes in the cell cytoplasm and in chronic infection the increase in the effectiveness of the therapeutic compound is related to its delivery to the nucleus (Zelphati et al. 1994).

Another type of liposome, known as sustained circulation liposome, is characterised by a significant increase in the half-life of the liposome. Within this group of liposomes, the so-called "sterically stabilised liposomes" are important. These incorporate polyethyleneglycol (PEG) at the surface of the liposome, which provides them protection against phagocytosis by mononuclear cells, prolonging the time of circulation of the drug delivery. Combined use of sterically stabilised liposomes and immunoliposomes elicits an important increase in the selectivity of anti-HIV drugs for lymphoid tissues. Studies with indanavir incorporated into sterically stabilised immunoliposomes have shown that these liposomes are very specific *in vitro* and studies performed in mice have demonstrated a greater accumulation of the drug in lymphoid tissues over at least 15 days after injection of the liposomes (Gagne et al. 2002; Desormeaux and Bergeron 2005).

Nanoparticles

Nanoparticles are stable, solid, polymeric particles with a size range between 10 and 1000 nm that are able to incoporate drug in their interior or bound to the polymeric matrix (Bender et al. 1996; Löbenberg et al. 1998; Von Briesen et al. 2000). They are formed of biodegradable polymers of natural or synthetic origin which are degraded *in vivo* either by an enzymatic route or a non-enzymatic action or a combination of both, giving rise to non-toxic products that are readily removed from the organism through the usual metabolic pathways. The natural polymers used are bovine serum albumin, human serum albumin, collagen, gelatine and haemoglobin, although their use is limited owing to their high cost and doubtful purity. Accordingly, it currently is more common to see the use of synthetic polymers, owing to their elevated biodegradability and biocompatibility, such as polyamides, polyamino acids, polyalkyl-α-cyanoacrylates, polyesters, polyorthoesters or polyurethanes (Jain 2000).

The nanoparticles of polymeric systems have been tested to improve the efficiency or antiretroviral drugs. Zalcitabine and saquinavir were formulated in nanoparticles and tested *in vitro* in HIV-infected human macrophages. In both cases, effective drug concentrations were achieved inside the cells. However, zalcitabine incorporated into nanoparticles did not show any improvement with respect to the free drug since this latter does penetrate macrophages. In contrast, saquinavir in nanoparticles elicited important improvements in antiviral activity in both chronic and acute infection (Bender et al. 1996). The distribution throughout the organism of AZT formulated in nanoparticles was studied after oral and i.v. administration to rats (Löbenberg et al. 1997, 1998). In both cases, encapsulation in nanoparticles implied an increase in bioavailability and also in the concentration of the drug in blood, brain and organs of the RES rich in macrophages, such as the liver, lungs, spleen and bone marrow in comparison with the drug in solution. Coating the particles with polysorbate 80 modified the nanoparticles distribution, the drug levels decreasing in liver and increasing in blood and in the brain. This is an advantage because the brain is one of the targets of the virus that is very hard to access with currently available antiretrovirals (Löbenberg et al. 1998). Nanoparticles constituted by complex of antisense oligonucleotides and their phosphorotioate analogues with protamine enhanced the cellular uptake of oligonucleotides and the inhibitory effect on HIV1 transactivation in primary human macrophages and Jurkat cells (Dinauer et al. 2004). Microparticles and nanoparticles have also been proposed as future delivery systems for DNA vaccines (Cui and Mumper 2003).

Low density lipoproteins (LDL)

Plasma LDLs are quasispherical endogenous nanoparticles with a diameter of approximately 22 nm, formed by a lipid nucleus surrounded by a phospholipid monolayer containing cholesterol and apoprotein B-100 (apo B) (Kader et al. 1998). Their metabolism involves internalisation by means of LDL receptors present on almost all cells of the organism, through the lipoprotein Apo B present in the LDL and in cells belonging to the monocytes-macrophage system by means of scavenger receptors (Mankertz et al. 1997; Hu et al. 2000; Von Briesen et al. 2000). Chemical modifications typical of the metabolism of Apo B—glycosylation, acetylation, derivatisation to aldehyde and oxidation alter the affinity of LDLs for scavenger

receptors of phagocytic cells. Owing to their lipid nature, they are useful for the transport of lipophilic drugs. Accordingly, the binding of anti-HIV drugs to modified LDLs is a strategy for transporting these substances to the inside of phagocytic cells (Hu et al. 2000).

Acetylated LDLs have been used as carriers for AZT to phagocytic cells. Owing to the hydrophilic nature of AZT, a prodrug—5′-*O*-13-oxamyristate-AZT- was used; this is more lipophilic and more effective than AZT. Cellular uptake was much greater than with the free drug both in mouse J774 macrophages and in human U937 monocytes (Hu et al. 2000). Flucytosine (FLT) and AZT bound to LDLs inhibited the replication of the virus in human macrophages but not in lymphocytes since the latter do not have scavenger receptors (Mankertz et al. 1996).

Peptides

Peptides have been proposed as carriers of drugs and genetic material because they are able to penetrate the lipid barrier of cell membranes and release their load of therapeutic agents intracellularly in the cytoplasm or nucleus. These peptides, known as protein transduction domains (PTD) or cell-permeable peptides (CPP) may be of natural or synthetic origin. Peptides for cell transduction usually contain lysine or arginine as the Tat from HIV1. The passage of peptides across the cell membranes is accomplished through a process of protein transduction, delivering the therapeutic agents inside the cell. The mechanism of protein transduction inside the cell is an electrostatic interaction with the plasma membrane, penetration by macropinocytosis and release to the cytoplasm and nuclei (Hyndman et al. 2004; Futaki 2005; Gupta et al. 2005; Noguchi and Matsumoto 2006).

The use of protein transduction for anti-HIV therapy involves the transduction of a protein which has been engineered with a target site for HIV protease. Therefore, this protein will only become activated in cells containing HIV protease but will remain inactive in uninfected cells (Vocero-Akbani et al. 1999). Antisense oligonucleotide or interfering RNAs (siRNAs) are also potential candidates to be used in combination with PTD to increase stability, enhance cellular uptake and improve anti-HIV therapy (Noguchi and Matsumoto 2006)

Fusogenic peptides have provided an improvement in gene transfection efficiency (Vaysse et al. 2000). Antisense oligonucleotides such as oligodeoxynucleotides and oligophosphorothioates coupled to an influenza-derived fusogenic peptide increase cellular delivery and antiviral activity *in vitro* using CEM-SS lymphocytes infected by HIV1. At the same time, the covalent attachment of the fusogenic peptide to the oligonucleotide increased its resistance to enzymatic degradation (Lochman et al. 2004). This fusogenic peptide was derived from the influenza virus haemagglutinin envelop glycoprotein (Remeta et al. 2002). Recently, it has been suggested that fusion peptides inserted into T cells membrane may inhibit antigen-specific T cell proliferation and cytokine secretion *in vitro* (Gerber et al. 2004; Quintana et al. 2005).

Tuftsin (L-threonyl-L-lysyl-L-prolyl-L-arginine) is a tetrapeptide located in the Fd fragment of the gamma-globulin molecule, which is recognized by specific receptors of phagocytic cells, mainly macrophages. As well as showing specific affinity for macrophages, the peptide tuftsin also has activator properties typical of phagocytic cells. Tuftsin may be used as carrier of anti-HIV1 drugs in AIDS therapy with potential clinical applications (Fridkin et al. 2005). The synthesis of conjugates of Tuftsin with anti-HIV1 drugs uses solution methodology (Fridkin et al. 2005). Conjugates of Tuftsin with 3′-azido-3′-deoxythymidine (AZT) inhibits reverse transcriptase activity and HIV1 antigen expression and increases the processing and presentation of antigens and the release of IL-1 in mouse peritoneal macrophages *in vitro* (Fridkin et al. 2005).

Studies addressing the cellular uptake of antisense oligonucleotides conjugated with cell-penetrating model peptides have proposed that the enhanced biological activity of antisense oligonucleotides after derivatization with membrane-permeable peptides may be related to an increased affinity for target structures such as nucleic acids or proteins as well as to an improved membrane translocation (Oehlke et al. 2002).

Another alternative is the use of macromolecular prodrugs, which are made by covalent linking of inert macromolecules with a drug, obtaining a molecular prodrug with a change in the pharmacokinetic properties of the conjugate with respect to the free drug. An example is the use of glycosylated macromolecular conjugates of antiviral drugs with a polyaspartamide (Giammona et al. 1998, 1999; Cavallaro et al. 2004). Macromolecular prodrugs of AZT with α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) allow a sustained action effect of the antiretroviral drug to be achieved (Giammona et al. 1999).

Biological delivery systems

Biological carrier systems are an alternative to the more classical non-biological carrier systems, such as liposomes or nanoparticles, which are increasingly used. Within the different biological carrier systems, of great interest are cells and cell ghosts, which are compatible systems from the biological point of view and which are able to provide the sustained release and

specific delivery of drugs and genetic material to tissues, organs and cells. Biological carrier systems such as, erythrocyte ghosts, bacterial ghosts viral vectors and more recently, genetically engineered stem cells can be suitably manipulated and loaded with drugs or genetic material, permitting specific drug delivery *in vivo* with therapeutic application in anti-HIV therapy (Lanao and Sayalero 2006).

Erythrocyte ghosts

Erythrocytes account for the greater part of cells in the blood and are the main transporters of oxygen to cells and tissues in the organism. When erythrocytes age, they undergo a series of catabolic changes that lead to a loss of cell flexibility. These changes favour cellular lysis in the circulation or phagocytosis by the RES, especially by the spleen, where the erythrocytes are attacked by lysosomal enzymes that lead to breakage of the cell membrane and the degradation of haemoglobin.

Erythrocytes are potential biocompatible vectors for different bioactive substances, including drugs and proteins. Erythrocyte ghosts can be prepared in different ways, usually by hypotonic dialysis (Millán et al. 2004). They find application as drug reservoirs that permit a sustained release of the drug to the organism or for selectively carrying the drug to the RES, especially the liver, spleen and bone marrow. This latter application is of special importance in anti-HIV drugs because they are able to improve the penetration of such drugs into phagocytic cells to a considerable extent.

To increase the phagocytosis of carrier erythrocytes a frequent strategy is to modify membrane proteins, as in the case of the transmembrane band 3 protein, with substances such a zinc, the peptide melitin, the dye acridine orange or the oxidising agents phenylhydrazine and diamine, followed by stabilisation with an agent that fosters cross linking such as bis(sulfosuccinimidyl)suberate (BS3). This favours opsonisation by IgG and C3b, increasing phagocytosis by macrophages (Magnani et al. 1992; Perno et al. 1997; Fraternale et al. 2003).

Erythrocytes display a series of advantages that make them suitable as carrier systems for shuttling drugs into phagocytic cells of the RES. Important aspects are that they are easily handled, they have high biological compatibility and stability and they provide the possibility of achieving a sustained action effect of the encapsulated drug. Erythrocytes also allow the encapsulation of peptides with biotechnological applications (Grimaldi et al. 1997; Bax et al. 1999; Magnani et al. 2002; Hamidi and Tajerzadeh 2003; Millán et al. 2004; Rossi et al. 2005).

The use of erythrocytes as carrier systems does however have some drawbacks: the rapid elimination of erythrocytes by the RES, which may lead to toxicological problems; the rapid leakage of certain substances from the erythrocyte, which hinders their access to the RES; difficulties in preparation and storage since they are biological carriers (Álvarez et al. 1995; Jain and Jain 1997; Moss et al. 2000; Sugai et al. 2001; Valbonesi et al. 2001; Millán et al. 2004; Rossi et al. 2005). One of the main therapeutic applications of carrier erythrocytes is in the field of anti-HIV therapy. Anti-HIV peptides such as nucleoside analogues successfully inhibit the replication of immunodeficiency viruses. Considering the importance of the monocyte-macrophage system in infection by HIV1, the specific delivery of these therapeutic peptides into macrophages, which act as an important reservoir for the virus, is of huge therapeutic interest.

The encapsulation of antiretroviral drugs in erythrocytes has been studied in some depth. The active form of ddCTP encapsulated in erythrocytes inhibits HIV replication in infected macrophages and protects uninfected cells from infection (Magnani et al. 1992, 1995). In *in vivo* studies, it decreased spleenomegaly, lymphadenopathies and hypergammaglobulinaemia, with no alterations in haematological parameters in mice and in cats (Magnani et al. 1992, 1995). Sometimes the encapsulation of prodrugs is used; this leads to the release of the drug since they are degraded by erythrocyte enzymes. This is the case of the dinucleotide AZTp2AZT encapsulated in erythrocytes, which is able to provide a controlled release of AZT over long periods of time (Benatti et al. 1996).

Glutathione is an antioxidant agent able to inhibit viral replication. Its encapsulation in erythrocytes could be very useful for the protection of macrophages challenged by HIV infection in combination with other antiretroviral drugs. The use of AZT as a protector of uninfected macrophages and lymphocytes, combined with fludarabine to eliminate infected lymphocytes, used alternatively, has afforded promising results in infected mice. However, the administration of GSH in erythrocytes together with AZT improved the efficiency of the treatment (Fraternale et al. 2001). Infected mice were treated with AZT + DDI and AZT + DDI + GSH-erythrocytes. The reduction in the content of proviral DNA in bone marrow and brain decreased significantly in mice treated with AZT + DDI + GSH-erythrocytes (Fraternale et al. 2002, 2003).

In patients with AIDS, it is common to find opportunistic viral infections, such as herpes simplex (HSV), which also invade macrophages and increase HIV replication. There are antiviral drugs such as adefovir (PMEA) or acyclovir (ACV) that inhibit the proliferation of both HIV and HSV. PMEA encapsulation in erythrocytes achieved a better inhibition of HIV and HSV in human monocytes/macrophages than free PMEA, with no signs of cytotoxicity (Perno et al. 1997). Prodrugs are also encapsulated, as dinucleotides, and these are converted into the active

form of the drug by endogenous enzymes. The encapsulation of bis-PMEA (Rossi et al. 2001) or ACVpPMPA (Franchetti et al. 2000) exerted a greater degree of protection against HIV and HSV and for longer periods of time than did the free drug.

Biological carrier systems in HIV gene therapy

Gene therapy for the treatment of AIDS is an interesting but as yet underdeveloped therapy that aims to replace CD4+ and other immune cells by cells genetically engineered to resist virus replication (Amado et al. 1999; Fanning et al. 2003). The success of gene therapy against HIV1 depends on good functioning of the different components of the immune system, such as the thymus or bone marrow and the use of an appropriate gene able to render the cell resistant to infection by HIV1. Antisense oligonucleotides and ribozymes can be targeted to specific sites within the HIV genome (Poeschla et al. 1996). The use of small RNA molecules that produce gene inactivation is a frequently used tool in gene therapy (Nielsen et al. 2005). In this field, antisense RNAs and ribozymes are designed to inhibit cellular or HIV RNA function (Lamothe and Joshi et al. 2000).

New gene therapy strategies based in combinatorial therapies for the treatment of HIV using series of RNA-based inhibitors have demonstrated to be effective in reducing viral loads. Recents papers demonstrate that a combined gene therapy strategy using antisense RNA and ribozymes using viral vectors to target different sites within the HIV1 RNA in a CD4+ T lymphoid cell line or CD34+-derived monocytes provides enhanced inhibition of HIV1 infection. (Li et al. 2005; Ramezani et al. 2006).

A promising gene for HIV gene therapy is RevM10. Rev protein is a virally encoded sequence-specific RNA-binding protein (Pollard and Malim 1998). The binding of Rev to the RER and the transport of RER containing the RNA to the cytoplasm is essential for viral replication. RevM10 is a dominant negative form of the Rev trans-activator protein that efficiently inhibits human immunodeficiency virus (HIV) (Gottfredsson and Bohjanen 1997; Veres et al. 1998; Hamm et al. 1999). Studies carried out in cultured T cell lines and primary T cells have shown that relatively high steady-state levels of RevM10 protein are required to achieve the inhibition of HIV replication (Plavec et al. 1997).

The use of nucleic acid vaccines offers a promising technique for the development of prophylactic or therapeutic vaccines based on the use of DNA plasmids to induce immune responses by direct administration of DNA-encoding antigenic proteins to animals and this is also suitable for the induction of cytotoxic T cells (Donnelly et al. 1997, Felnerova et al. 2004; Lanao and Sayalero 2006).

Currently in the field of gene therapy for the prevention and treatment of HIV/AIDS gene carriers are being developed that encapsulate and protect the nucleic acid and selectively release the vector/nucleic acid complex to the target tissue so that the genetic material will later be released at cellular level. In practice, there are three ways to achieve this aim. The first is through the use of modified viruses containing the genetic material of interest. The second alternative is to use living cells modified genetically, such as stem cells, to deliver transgenic material into the body. The third alternative is the use of bacterial ghosts that encapsulate the genetic material and allow its specific delivery to phagocytic cells (Lanao and Sayalero 2006).

Viral vectors as gene delivery systems. Attenuated or modified versions of retroviruses, adenoviruses, lentiviruses, Sendai virus, herpes virus, adeno-associated viruses and poxviruses can all be used as carriers, with different therapeutic applications, although their use has been questioned (El-Aneed 2004; Tomanin and Scarpa 2004; Klink et al. 2004). The main advantage of the use of viruses as gene delivery vehicles is their high transduction efficiency *in vivo*. Retroviral transduction efficiency has been enhanced using ultrasonic standing-wave fields to facilitate the retroviral transduction rate (Lee and Peng 2005). However, gene delivery systems viruses have some drawbacks since they only deliver genes to cells during mitosis and they are sensitive to lysis by complement when they are administered to patients. Furthermore, viruses may mutate; they are limited in the size of gene they can carry and they may give rise to immune complications (Falkner and Holzer 2004; Klink et al. 2004).

Delivery systems based on the use of viruses as carriers have recently found application in the fields of HIV vaccines (Humeau et al. 2004; Excler 2005; Duerr et al. 2006). Poxviruses are a heterogenous group of DNA viruses that are potentially safe because they are unable to replicate in humans. Poxviruses have shown their ability to induce mucosal immune responses against foreign expressed antigens, which is important for the development of vaccines against mucosal pathogens, as happens with HIV (Gherardi and Esteban 2005; Moroziewicz and Kaufman 2005). Recent clinical trials in HIV1-exposed infants using recombinant modified Ankara virus vaccinia (MVA) as a vector to deliver recombinant HIV type 1 (HIV1) antigens (MVAHIVA) using HIV1-specific CD8+ cell responses suggest that stimulation with the MVAHIVA may be useful in the evaluation of vaccine receivers (Slyker et al. 2005). Recent clinical trials in healthy uninfected adults using canarypox ALVAC-HIV vaccines have revealed high reactogenicity associated with an increased dose of the vaccine

(Goepfert et al. 2005). Clinical trials and experiments carried out in mice and macaques using recombinant fowl pox virus vaccines directed against HIV1 subtype B, against HIV1 subtype AE, or SHIV have shown that the relevant proteins are expressed (Coupar et al. 2006).

Adenovirus type-5 (Ad5)-based vaccines produce an adequate T cell immune response in baboons and monkeys, although the hepatocellular tropism of this viral vector limits their safety (Casimiro et al. 2003; Xin et al. 2005). Adequate immune responses were recently obtained in macaques using an adeno-associated virus vector vaccine (Johnson et al. 2005).

Other viruses such as alpha-viruses, flaviruses, rhabdoviruses, myxovirus, paramoxyviruses or picorna virus may be used as potential vaccine vectors for anti-HIV therapy (Figure 2) (Excler 2005).

Recently, the "intracellular immunization" strategy has been tested. This is based on gene transfer using a spleen necrosis virus (SNV) as a retroviral vector with potential applications in future human gene therapy applied to anti-HIV1 treatment. *In vitro* experiments using human haematopoietic cells demonstrated the reduction of HIV1 replication by the SNV retroviral-vector carrying therapeutic transgenes in a range of 4–10-fold in T-lymphocytes, primary human PBMCs and macrophages (Marusich et al. 2005).

Stem cells. Another alternative is to use living cells modified genetically, such as stem cells, to deliver transgenic material into the body. The use of stem cells as delivery systems is a novel and attractive technique in the field of gene therapy in which the cells of the patients themselves are genetically engineered in order to introduce a therapeutic transgene and then used to deliver the genetic material (Becker 2005; Lanao and Sayalero 2006). Haematopoietic stem cells may be used as a drug delivery system of antiviral genes with a view to reducing HIV1 replication. Considering the affinity of primitive CD34+ haematopoietic stem cells for HIV host cells, stem cells can be collected and transduced *in vitro* by spinoculation (O'Doherty et al. 2000) with a retroviral or a lentiviral vector (Bai et al. 2003; Brenner and Malech 2003) expressing the trans-dominant mutant rev protein RevM10, which inhibits HIV1 replication. Autologous engineered haematopoietic stem cells administered *in vivo* allow the delivery of genetic material to target cells such as monocytes or macrophages, suppressing HIV1 replication (Amado et al. 1999; Mautino et al. 2001) (Figure 3). The use of haematopoietic stem cells has allowed antiviral genes to be introduced into both T cells and macrophages for the treatment of AIDS (Bonyhadi et al. 1997; Morel et al. 1999; Chan et al. 2005; Van Griensven et al. 2005). *In vitro* and *in vivo* studies have shown the potential usefulness of haematopoietic stem cells for anti-HIV1 therapy (Siapati et al. 2005). Preliminary experiments *in vivo* after reconstitution of human bone marrow implanted in mice with transduced haematopoietic stem cells with RevM10 have revealed an increase in the myeloid progenies expressing RevM10 (Su et al. 1997).

Clinical studies performed in paediatric AIDS patients using bone marrow haematopoietic CD34 + stem cells isolated and transduced by a retroviral vector as carriers of an RRE anti-HIV1 gene and reinfused in the patients showed that gene-containing leukocytes in the peripheral blood were seen only at a low level and only at the start of treatment (Kohn et al. 1999; Bauer et al. 2000). More recent clinical trials carried out by the same group in 2 paediatric HIV1 patients using two retroviral vectors, one encoding a dominant-negative REV protein (REVNL3-huM10) and another encoding a non translated marker gene (REVNL3-FX), showed that following cell transduction and reinfusion of the CD34 + bone marrow stem cells, gene-marked cells were present at low levels and persisted for only a few months. In one of the patients, the reappearance of

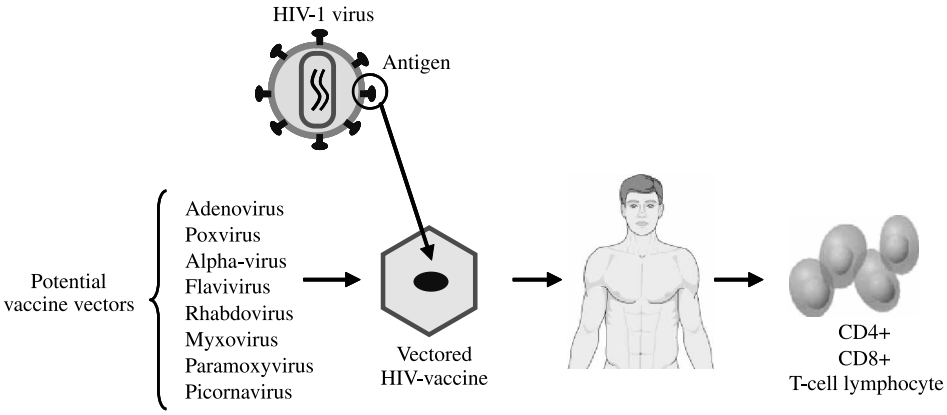

Figure 2. Viral vectors for HIV-1 vaccines.

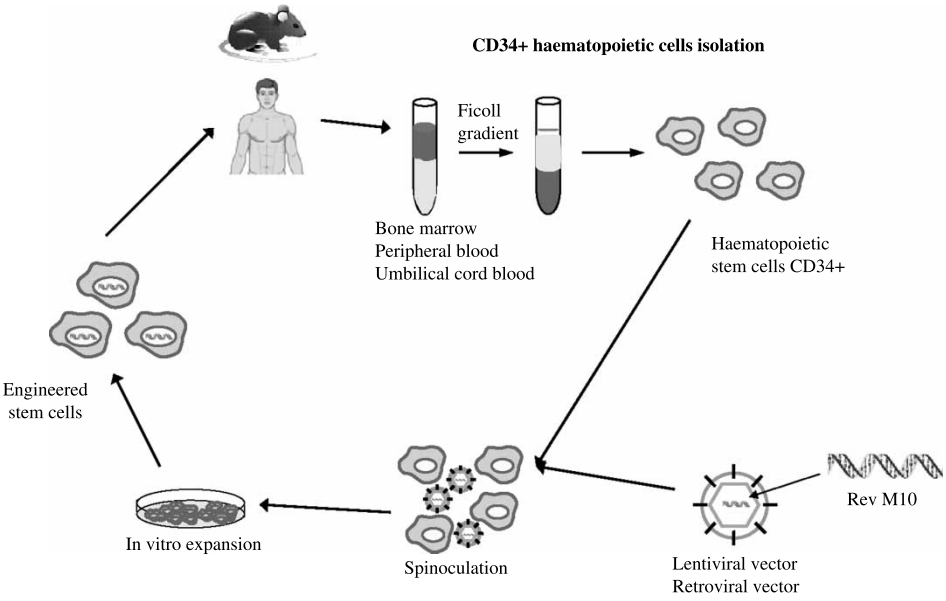

Figure 3. Use of engineered haematopoietical stem cells for anti-HIV therapy.

peripheral blood mononuclear cells containing the huM10 gene suggested selective survival for the peripheral blood cells containing this gene (Podsakoff et al. 2005).

Although the use of stem cells to inhibit HIV is a promising technique in the field of gene therapy, there are some drawbacks that remain to be solved. Examples are altered haematopoiesis and thymopoiesis during HIV1 infection, or the need to increase the amount of transduced haematopoietic stem cells, among others (Van Griensven et al. 2005).

Bacterial ghosts. Bacterial ghosts are intact, non-living, non-denatured bacterial cell envelopes devoid of cytoplasmic contents that are created by lysis of bacteria but that maintain their cellular morphology and native surface antigenic structures (Lanao and Sayalero 2006). Bacterial ghosts are produced by protein E-mediated lysis of Gram-negative bacteria (Marchart et al. 2003). Bacterial ghosts allow the encapsulation of drugs, proteins and therapeutic DNA or RNA, allowing specific delivery to different tissues and cell types and they have a good capacity for being captured by phagocytic cells, and antigen-presenting cells such as dendritic cells. The main disadvantages of this type of delivery system are the possibility that they might revert to being virulent, the possibility of lateral gene transfer, the stability of the recombinant phenotype and pre-existing immunity against the vector used (Tabrizi et al. 2004).

Bacterial ghosts are a promising delivery system for DNA vaccines (Ebensen et al. 2004; Tabrizi et al. 2004; Walcher et al. 2004; Mayr et al. 2005). Although there is little experience in the use of bacterial ghosts in HIV1 therapy, in experiments carried out using animal models with piglets, mice and rabbits using bacterial ghosts of *K. pneumoniae*, *S. typhymurium* and *E. coli* as carriers of HIV1-RT and HIV1 gp41 as antigen targets it was observed that these ghosts induced humoral and cellular immune responses (Szostak et al. 1993, 1996; Walcher et al. 2004; Mayr et al. 2005).

Concluding remarks

In the past ten years, many advances have been made in the development of both non-biological and biological carrier systems in anti-HIV therapy. In the field of non-biological carrier systems, liposomes have been developed to carry drugs such as stavudine, dideoxycytidine or AZT that allow the specific delivery of these drugs to infected macrophages. Assays have been made in immunoliposomes containing antisense RNA. Advances have also been made in the field of nanoparticles, LDL and peptides containing anti-HIV drugs and genetic material destined for gene therapy. However, despite such progress most studies carried out have been limited to the *in vitro* situation or in experimental animals and there is very little clinical information about this type of carrier in humans. On the other hand, important advances have been made in the use of biological carrier systems for the treatment of HIV using erythrocyte ghosts, bacterial ghosts, viral vectors and engineered stem cells.

Of special interest is the progress made in the field of HIV gene therapy through use of autologous stem cells and viral vectors. These have led to important milestones in the development of HIV vaccines in clinical assays carried out all over the world. In the near future, it would be desirable to see a boost in clinical trials with new carrier systems in HIV patients.

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