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Retroviral and Lentiviral Gene Therapy for Autoimmune Disease

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Autoimmunity is a pathological state in which the body's own tissues are the target of immune attack. This can arise as a result of a number of factors such as antigen mimicry, failure in deletion of autoreactive T cell clones or aberrant cytokine production. Autoimmunity encompasses a broad range of diseases such as rheumatoid arthritis, insulin-dependent diabetes mellitus, systemic lupus erythematosis and multiple sclerosis. Current therapies focus on trying to alleviate symptoms; for example, the injection of insulin by diabetics and the administration of steroids to reduce painful inflammation in rheumatoid arthritis. However, these fail to get to the root of the problem, as they do not correct the fundamental immune defects that lead to pathology. To achieve this goal, it has been necessary to understand much of the biology of antigen presentation, lymphocyte activation and the effects of cytokines. Although immune responses can be manipulated in vitro and in vivo by addition of recombinant cytokines, this is not a viable therapeutic approach. Systemic delivery of cytokines has had catastrophic effects in the past [1]; it is obvious that administration of cytokines, or factors interfering with cytokine function, needs to be on a local basis and, if possible, be tightly regulated.

Gene therapy offers a potential solution to some of these problems. However, gene therapy is a technology still in its infancy. What techniques are available for developing gene therapies for autoimmune diseases, and how have these contributed to our understanding of the biology of the immune system?

Table 1. A comparison of current vector systems for gene transfer

	Insert size	Titer	Integration	Sustained expression	In vivo delivery	Non-dividing cells transfected
Liposomes	>20 kb	N/A	No	Transient	Variable	Yes
Plasmid DNA injection	>20 kb	N/A	No	Only in muscle	Poor	Yes
Adenovirus	8 kb	10^{11}	No	Transient	High	Yes
Adeno-associated virus	<4 kb	10^{9}	Yes (wild-tye virus)	Transient	High	Yes
Retrovirus	8 kb	10^{7}	Yes (random integration)	Variable	Variable	No
Lentivirus	8 kb	10^{6}	Yes (random integration)	Variable	?	Yes

Vectors

Gene therapy vectors can be divided into nonviral and viral systems. These are summarized in table 1. Nonviral systems preceded viral systems, and were developed largely as methods of introducing DNA into cells in vitro. This review will only briefly discuss those used in vivo.

Liposomes made from artificially synthesized lipids have been used in vitro since the 1960s and have been used in vivo in a number of trials designed to target genes to the respiratory tract [2]. There are two classes: cationic liposomes, which form complexes with negatively charged DNA, and pH-sensitive, or negatively charged liposomes, which entrap DNA in an internal aqueous pocket. The latter class of liposomes is far more efficient at gene delivery than the cationic lipids. However, both classes of lipids show poor delivery efficiencies, with transient gene expression and the lipids themselves exhibiting varying degrees of cytotoxicity, coupled with inactivation by serum proteins.

The observation that naked DNA could be injected into striated muscle and be expressed opened up the field of DNA vaccination [3]. The advantage of this is that plasmid DNA is easy to produce, and can potentially deliver large fragments of DNA. Despite the fact that enough protein can be expressed to generate an immune response, gene expression is transient in all tissues other than muscle, the transgene does not integrate with the host DNA, and only around 1% of muscle cells express the gene. Attempts to extend DNA injection to other tissues have failed.

It is clear that nonviral techniques as they currently stand are poor choices of vector for gene therapy, particularly if it is desired to target the immune system, which would be necessary in the treatment of autoimmunity. Fortunately, nature has provided us with highly efficient vehicles for delivering foreign genes into cells: viruses.

Viral Vectors

A number of different virus groups have been utilized in gene transfer protocols. This review will briefly describe the adenovirus and adeno-associated virus (AAV) systems, which have both been utilized in autoimmunity studies.

Forty-seven serotypes of adenovirus are currently known. Adenoviruses infect the respiratory tract, and are one of the causes of cold-like illness, though they have a wide tissue tropism [4]. The genome is complex, though in gene transfer vectors the majority of the regions involved with replication (E1A) and pathogenesis (E3) have been removed to create 'gutless' adenoviruses. Despite high titers (10^{10}–10^{13} infectious particles per milliliter), and the ability to carry an insert of up to 8 kb in size extremely efficiently to both dividing and quiescent cells, adenoviruses are strongly immunogenic. As the vast majority of the population has been exposed to adenoviruses, some thought has been given to using animal adenoviruses in human gene therapy, though repeated administration may be impossible due to strong immunogenicity. Gene expression is transient, and the vector does not integrate into the host DNA. Adenoviral vectors are also time-consuming to prepare, requiring plaque purification to eliminate the presence of wild-type virus.

The AAV are parvoviruses with a single DNA strand genome of 4–5 kb [5]. AAV are satellite viruses of adenovirus, requiring coinfection with adenovirus to replicate. Although AAV are small, their great advantage is that the wild-type virus selectively integrates into the same site in chromosome 19 by virtue of the inverted terminal repeats in the viral genome. This specific integration eliminates any fears concerning oncogenesis arising from random insertion into the chromosomes. Unfortunately, recombinant AAV does not show this specificity; this is currently a major research focus. Vector production also requires coinfection of the packaging cell with wild-type adenovirus, which must be removed from the final vector preparation. This is usually achieved by density gradient centrifugation or heat inactivation, though these methods are by no means foolproof.

These concerns have led many to consider that the systems with the most promise for human gene therapy are likely to be the retroviruses and lentiviruses, which will now be discussed.

Retroviral Vectors

Retroviruses consist of a diploid positive (sense) single-stranded RNA genome of between 3.5 and 10 kb in length. The genomes are contained within a protein capsid, which is itself within a lipid envelope. Upon cell entry, the RNA genome is reverse transcribed by a viral polymerase, with the resulting double-stranded DNA genome becoming inserted within the host cell's DNA to form the provirus. The proviral DNA is transcribed to generate viral mRNAs (which are themselves translated to give proteins for manufacturing new virions), and the next generation of viral genomes. The insertion step, which is an essential part of the retroviral life cycle, renders retroviruses a stable part of the host cell genome. The ability to insert into the host cell chromatin is a valuable feature of retroviral vectors, as it ensures partition of the transgene to all daughter cells of the original target.

By far the most widely used retrovirus in gene transfer technology is the Moloney murine leukemia virus (MMLV). The MMLV genome is very simple, consisting of two long terminal repeats (LTRs) which contain sequences important in initiating DNA synthesis and transcription, flanking three protein-encoding regions, gag, pol and env. The gag region encodes the capsid protein while the reverse transcriptase and integrase functions are encoded by the pol region. The env region encodes the envelope protein. MMLV envelopes are classified by the host range they confer upon the virus. The ecotropic envelope allows infection of only murine cells. Amphotropic and polytropic envelopes allow infection of both murine and nonmurine cells, whilst xenotropic envelopes only infect nonrodent cells. Between the 5'LTR and the gag open reading frame (ORF) a region of conserved RNA secondary structure is found which serves as the packaging signal. This sequence is needed to ensure encapsidation of the viral genomes.

Retroviral transfer DNA vectors are plasmid DNAs consisting of two retroviral LTRs flanking an internal region containing the packaging signal and the gene that it is desired to deliver to the target cell (fig. 1). A second ORF can also be incorporated for expression of a reporter gene by including an internal ribosome entry site or an internal promoter. This allows two separate cistrons to be translated from a single mRNA. How is the RNA from this construct packaged into new virions? The retroviral vector transfer construct must be transfected into a packaging cell line that provides the gag, pol and env proteins necessary for the formation of intact virions. (Remember that the transfer construct includes the packaging signal, and therefore only requires addition of the viral proteins in *trans* in order to be encapsidated.) Early packaging cell lines simply contained retroviral proviruses, complete except for having the packaging signal deleted, from which the viral proteins

were generated [6]. However, only a single recombination event is required in such systems to generate replication-competent virus, which will not contain the gene it is desired to deliver, and which could be potentially harmful in a therapeutic context. In order to reduce the chances of replication-competent virus being formed, later cell lines expressed the retroviral proteins off different plasmids [7, 8]. Although such cell lines did not produce replication-competent virus, vector was only produced at high titers for short periods. It is desirable to be able to generate cell lines that stably produce high titers of virus for long periods of time. The Phoenix cell line (fig. 1a) achieves this by including selectable markers that are coexpressed with the retroviral proteins, enabling producer cells to be chosen by antibiotic and FACS selection for stable, long-term expression of the gag, pol and env proteins [Achacoso PA, Costa GL and Nolan GP, in preparation]. Titers of up to 10^7 virions per milliliter can readily be achieved from transient transfection of the transfer vector. The Phoenix cell lines can generate either ecotropic or amphotropic virus, depending on the envelope packaging vector used. It is also possible to use foreign envelope proteins to generate pseudotyped virions with different targeting properties (discussed in more detail below). By incorporating the vesicular stomatitis virus glycoprotein into the retroviral envelope in this fashion, it is possible to generate a retrovirus that is capable of surviving concentration by ultracentrifugation, which may be necessary for producing therapeutically usable titers [9]. Inclusion of a nonretroviral envelope gene can also reduce the likelihood of a recombination event between the packaging and transfer constructs. In a further modification, it is possible to include elements in the transfer vector that enable it to become established as an episome [10]. Selection with appropriate antibiotics allows the maintenance of cells that consistently produce high titers (approximately 10^7/ml) of virus over a period of months, starting from only a week after the initial transfection.

In the most basic retroviral vectors, transcription is driven from the promoter and enhancer elements in the 5′ LTR. Although retroviral LTRs are extremely powerful constitutive promoters, such uncontrolled expression is not always desired. Therefore it is possible to construct self-inactivating (SIN) vectors, in which the 5′ LTR is disabled during the formation of the provirus [11]. This is achieved thanks to the reverse transcription mechanism of proviral formation, in which the 3′ LTR of the RNA genome forms the template for the 5′ LTR of the provirus. The parent template transfected into the packaging cell contains an intact 5′ LTR to allow generation of RNA genomes for encapsidation, but the 3′ LTR contains inactivating deletions. SIN vectors such as this have been used to allow inducible expression, with transcription being controlled by the presence or absence of a small molecule, such as the antibiotic tetracycline [12, 13]. Alternatively, tissue-specific promoters have

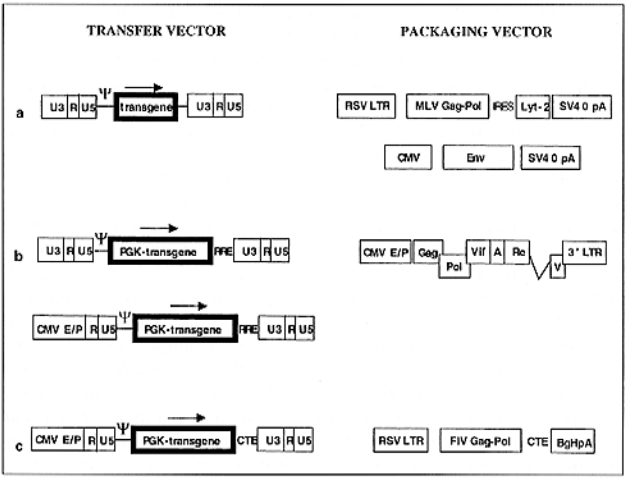

Fig. 1. A comparison of retroviral and lentiviral vectors. These systems comprise of a transfer vector, which encodes the RNA that will form the vector genomes. The transfer vector is transfected into a packaging cell line expressing the proteins needed for forming vector particles. *a* The transfer vector used in MMuLV-based retroviral vectors consists simply of the transgene flanked by the retroviral LTRs (consisting of regions U3, R and U5). The packaging signal, Ψ, is also included to ensure RNA encapsidation. Variations of this basic transfer vector include the addition of a second open reading frame, which is translated from an internal ribosome entry site (IRES). Alternatively, transcriptional control can be removed from the LTR, and given to an internal promoter, such as the PGK (phosphoglucokinase) promoter. This is achieved by deleting the U3 region from the 3′ LTR, thus generating a self-inactivating (SIN) vector (see main text). In the latest packaging cell lines, including the widely used Phoenix line, the gag-pol and env open reading frames are situated on different plasmids, under the control of nonretroviral LTRs in order to reduce the chance of homologous recombination and the generation of replication-competent virus. In the Phoenix line, the Lyt-2 surface marker is included downstream of an IRES element to allow selection of the producer cell by FACS. *b* The transfer vectors of the second-generation FIV vectors are similar to those of the MMuLV systems. Important differences to note are the inclusion of an internal promoter (PGK), and the rev response element (RRE). The RRE is required to ensure export of full-length RNAs from the nucleus. These vectors gave titers of around 10^4 IU/ml, but substituting the 3′ U3 region with the CMV enhancer and promoter (CMV E/P) increased titers to around 10^6 IU/ml. The packaging vector consists of nearly all the FIV genome. The 5′ LTR has been removed and replaced with the CMV enhancer and promoter (CMV E/P). The envelope gene has been completely removed, to fuse the two

been inserted to allow expression only in a particular subset of the cells infected [14]. This approach is described in detail later.

Although retroviruses have many advantages, their main disadvantage is that they can only transduce cells that divide shortly after infection. A large number of cell types of interest as potential therapeutic targets, such as neurons, muscle cells and hematopoietic progenitors are therefore refractory to retroviral infection. Lentiviral vectors may offer a solution to this problem.

Lentiviral Vectors

Wild-type lentiviruses infect macrophages and lymphocytes, cell types that are generally not dividing. Clearly, lentiviruses must have evolved a mechanism to deliver their genetic material through the nuclear membrane (which disappears during cell division, allowing simple retroviruses access to the chromatin) to be integrated into the host DNA. It has been shown in vitro that HIV can infect growth-arrested cells. The complete mechanism has yet to be fully understood. The nucleoprotein complex that is delivered to the cytoplasm consists of the viral RNA plus the gag matrix, vpr and integrase proteins [15, 16]. Though nuclear localization signals are present, the relative contribution of these proteins is not known.

The first generation of lentiviral systems attempted to generate replication-defective HIV vectors, though these vectors had low infectious titers and were restricted to CD4+ cells [17, 18]. They were also prone to recombination with the packaging constructs to produce replication-competent HIV. These vectors were improved by pseudotyping with the envelope of other viruses [19]. This allowed efficient infection on nondividing human cells in vitro, as well as sustained long-term expression in the central nervous system of rats [20]. Vectors were generated in much the same way as the retroviral vectors described

Rev gene introns (Re and V) into one. In wild-type virus the envelope gene lies between the two Rev introns. The accessory protein Vif remains, as does open reading frame A, and the 3′ LTR which is required for polyadenylation. The envelope protein is supplied on a separate plasmid (not shown). The large percentage of FIV sequence does raise the risk of recombination between transfer and packaging vectors. *c* In an attempt to reduce the degree of homology between the transfer and packaging vectors, third-generation vectors were developed. In the packaging vector, the only FIV-derived sequences are those encoding the gag and pol proteins. The RRE has been replaced with the nonlentiviral CTE, and polyadenylation is provided by the appropriate signal from the bovine growth hormone gene. The envelope protein is supplied on a separate plasmid (not shown). Titers are dependent upon the position of the CTE. Infectivity increases as the CTE is moved towards the 3′ end of the transfer vector, to a maximum titers in the order of 10^6 IU/ml.

earlier, with packaging cells containing two packaging constructs, encoding virus proteins, being transfected with the transfer construct encoding the transgene along with the *cis*-acting sequences required for encapsidation and reverse transcription. The evolution of lentiviral packaging systems has paralleled that of retroviruses. Initial packaging systems required the transient cotransfection of packaging and transfer constructs, giving only a short period of high-titer vector production, plus the increased possibility of recombination. Early attempts at generating stable producer lines, including the use of inducible systems, resulted in poor titers [21].

There have been considerable improvements to lentiviral vectors since these initial studies. It has been determined that fewer sequences are required for HIV-derived vectors to function than are needed for the replication and pathogenesis of wild-type HIV. Elimination of the four accessory genes vpr, vif, vpu and nef gives functional vectors with much improved biosafety [22, 23]. A further important step in improving safety was the elimination of the tat gene. The tat protein is an extremely potent, virally encoded transcriptional activator. Cells stably expressing HIV structural proteins have a low dependence on tat, though such cells only produce vector titers in the order of 10^4 infectious particles per milliliter [24]. More recently, it has been demonstrated that tat function can largely be replaced by constructing vectors with the CMV promoter substituting for the U3 region of the LTR [23]. It has also been possible to dispense with the promoter functions of the viral LTR completely and generate SIN lentiviral vectors in much the same way as the retroviral vectors described earlier [25].

Why can these genes which are indispensable for wild-type HIV be discarded from a lentiviral vector? It is important to remember that virus production only occurs in the packaging cell, and that infection by a vector is not the same as infection by HIV. Pseudotyped vectors infect cells different from the natural targets of HIV, and via different routes. Wild-type HIV infects by fusion with the plasma membrane, whilst HIV-VSV vectors infect via the endocytic pathway, which has a significant effect on the biology of infection, dispensing with the requirement for nef [26]. However, the choice of the target cell will have a bearing on the accessory proteins required by the vector. For example, vpr is required for the transduction of hepatocytes and macrophages, but can be dispensed with when infecting neurons [19, 22, 23]. There is evidence that wild-type HIV and HIV-derived vectors do not productively infect resting lymphocytes and macrophages. This results from blocks at the levels of reverse transcription and nuclear import. However, if cells are activated, but not necessarily dividing, then they do become infectable. These observations have important implications for the use of such vectors in autoimmune therapy.

There is considerable concern surrounding the use of HIV as the basis of a gene therapy system because of the possibility of the vector being given to a patient infected with wild-type HIV. This could lead to the propagation of the vector (as the patient's HIV-infected cells could act as packaging cells), or the generation of new, potentially hazardous, recombinant viruses. Therefore, there has been considerable interest in nonprimate lentiviruses, specifically feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV) (fig. 1b, c). FIV is an attractive choice as there is no evidence of human infection, despite veterinarians having been bitten and scratched by infected animals. But where is the block to infection of human cells? Although the FIV envelope can mediate infection of human cells, the transcriptional activity of the LTR remains low in nonfeline cells. Promoter substitution allowed high level expression of vector-encoded transgene in human cells [27]. The FIV genome, in common with that of other lentiviruses, contains many splice acceptor and donor sites. RNA processing is an important part of the lentiviral life cycle, being necessary for generating appropriate transcripts for translation of the various reading frames. In order for full-length genomic vector RNAs to be efficiently exported to the cytoplasm for packaging, it is necessary to include appropriate RNA signal sequences. Lentiviruses encode a protein known as rev, which binds to genomic RNA, and prevents its processing. Although the FIV rev and its response element (RRE) are active in human cells, substituting them with the HIV homologs gave greater activity. However, in an effort to limit the amount of lentiviral sequences in the transfer vector, a nonlentiviral sequence with analogous function to the RRE, known as the cytoplasmic transport element (CTE) has been used in the FELIX system, and shown to function efficiently [Curran M.A., Kaiser S.M., Achacoso P.A., Nolan G.P., in preparation]. In addition, it has been shown that vectors generated with the FELIX cell line have insignificant cross-packaging with wild-type HIV, an observation of vital importance from the view of biosafety.

Can FIV vectors be used to infect cells of the immune system which may be of interest in the treatment of autoimmune disease, such as monocytes, dendritic cells and lymphocytes? Curran et al. have demonstrated that vectors produced with the FELIX system can efficiently infect primary human smooth muscle cells, hepatocytes and dendritic cells. Unlike HIV, the FIV vectors do not require the vpr accessory protein to infect hepatocytes.

Do lentiviral vectors offer any real advantages over retroviral vectors in the treatment of autoimmune disease? To answer this, we must consider what cell types will be targeted. As will be seen in the later parts of this review, retroviral vectors have been used with considerable success in many systems. However, there are other cases in which lentiviruses are clearly more efficient,

such as the infection of hematopoietic stem cells and professional antigen-presenting cells, populations of great interest to the immunologist.

Targeted Retroviral and Lentiviral Vectors

One fear of critics of gene therapy is that the vector systems being used may not have enough tissue specificity if administered in vivo, resulting in the delivery of genes to the wrong tissues with possibly harmful effects. The current generation of packaging cell lines allows different envelopes to be used to generate pseudotyped viruses with altered specificity. At its crudest level, pseudotyping involves the substitution of an entirely novel gene in the envelope packaging vector, such as the vesicular stomatitis virus glycoprotein (described above) or the gibbon leukemia virus envelope, both of which have been used in the MMLV system. However, it would be desirable to engineer novel envelope proteins for highly specific targeting against a particular cell type.

The retroviral envelope consists of two polypeptide chains, a transmembrane fusogenic domain, which is linked by disulphide bridges to a large extracellular domain which interacts with the virus receptor on the target cell. Towards the N-terminal of the extracellular domain are variable regions, differences in which can alter virus tropism from ecotropic to amphotropic, and vice versa. Following this observation, many groups have tried to remove these regions and replace them with ligands for particular receptors or with single chain antibody variable region fragments (scFv) reactive against cell surface receptors in order to change virus tropism. Kan's group has inserted whole proteins such as erythropoietin and human heregulin into the N-terminal of the MMLV ecotropic envelope extracellular domain [28]. Although viruses bearing these modified envelopes were able to infect human cells carrying the appropriate receptors (erythropoietin receptor and human epidermal growth factor receptor, respectively), titers were extremely low (in the order of 10^3 infectious particles per milliliter). The specificity of the infection is also questionable given that no competitive inhibition assays were attempted with native EPO or heregulin. No progress in either of these systems has been published, casting doubts onto the validity of this approach to retroviral retargeting. Similar problems have also been experienced with the use of scFv chimeric envelopes. Retroviruses bearing such envelopes can bind a solid matrix onto which the scFv ligand is affixed, and the resulting bound virus could infect 3T3 cells (possibly via endocytosis of the virus-antigen complex rather than true retroviral infection) [29]. However, direct infection of the target cell by the virus has not been shown. If the virus can bind the appropriate ligand, why does infection not take place? It is thought that addition of these extra

domains to the envelope disturbs the structure to such an extent that though receptor-ligand binding occurs, the fusogenic domain cannot function.

Obviously, it is impossible to add large domains to the envelope and expect it to function. However, it may be possible to add small peptides, as there are only a few amino acid changes between the ecotropic and amphotropic envelopes. Where should these peptides be inserted? Analysis of the avian leukosis virus (ALV) envelope has revealed four small regions at the N-terminus of the extracellular domain which appear to be involved in receptor binding. Could it be possible to change the sequence of one or more of these regions in order to allow ALV to infect mammalian cells that are normally refractory to ALV? A 16-amino-acid peptide containing the argine-glycine-aspartic acid (RGD) integrin-binding motif was inserted into each of the four regions. Only the first two regions could accommodate the RGD peptide and still produce correctly processed envelope. Viruses carrying these mutant envelopes were able to bind to mammalian cells, though the viruses required deglycosylation treatment in order to allow infection. This infection is clearly specific, as it could be inhibited by preincubation of the target cells with the RGD peptide [30, 31].

Although these data are encouraging, they are still not ideal, as the virus preparations required deglycosylation. Is it possible that the peptide is not occupying the optimal position to allow retargeting? How can we find the best place for making the modification to the envelope without having to resort to cloning and testing hundreds of different envelopes? Recent work has suggested that to answer these kinds of questions it is best to let nature do the work. La Barge et al., [in preparation] have randomly scanned in an oligonucleotide encoding the integrin binding motif RGD through the ecotropic receptor gene. This was achieved by performing a limited digest with DNase to cut each plasmid on average once, then religating with an oligonucleotide encoding the RGD peptide. This produces a library of mutant envelopes where the RGD peptide should be found at every possible position. Like the ALV envelope described above, the ecotropic receptor cannot mediate the infection of human cells. A selection procedure was established where the library was allowed to infect human 293T cells which were also expressing retroviral gag and pol proteins. Only viruses carrying an envelope in which the RGD motif was optimally placed were able to infect the 293T cells, and be propagated further, thanks to the gag and pol proteins supplied by the target cells. Through two successive rounds of selection a pool of viruses with modified envelopes was formed. On infection of 293T cells the resulting virus was found to be more infectious than viruses carrying the naturally occurring amphotropic envelope. PCR analysis revealed three different major populations of envelope, ranging from almost full length to having nearly all the

extracellular domain deleted. Further investigation has revealed that these different envelope mutants must be found on the same viral particle, and complement each other in *trans* in order to allow infection. It is interesting to observe that the retroviral envelope is tolerant of large deletions (provided that complementary envelopes are available to assist infection) while at the same time not supporting the addition of large domains. This suggests that it is important not to block access of the fusogenic domain to the target cell envelope with bulky extra polypeptide domains.

Can retroviral vectors be directly injected into the body and find their way to their targets? Initial work in mice using MMLV vectors indicated that care must be taken in setting up experimental systems in considering the strain of mouse to be used [32]. Different mouse strains carry different endogenous retroviruses, some of which express circulating envelope proteins that can interfere with retroviral infection. This is an important point to consider in human gene therapy. Will there be any proteins from endogenous retroviral elements (which make up approximately 1% of the genome) which may interfere with vector infection? Although it has been clearly shown that retroviral infection can occur around the site of injection, is it possible to get infection at distant sites? This is a concern for autoimmune therapies as it may be desired to target professional antigen-presenting tissues such as the spleen and lymph nodes. Although infection may not occur at a distant site, migratory cells may carry the transgene to where it is required. Song et al. demonstrated that the majority of infiltrating cells around the site of retroviral injection in the muscle of mice were of the monocyte/macrophage lineage [33]. These then presumably migrated to the spleen, as the splenic dendritic cell fraction was found to contain proviral DNA and to be expressing the virally encoded transgene. This transgene was chicken ovalbumin; isolated splenic dendritic cells were shown to specifically stimulate activation of chicken-ovalbumin-reactive T cells. Dendritic cells are potentially extremely important targets for the delivery of factors to induce tolerance to autoantigens by interfering with antigen presentation.

Autoreactive T cells may themselves serve as delivery vehicles for transgenes [34a]. It has been shown in experimental allergic encephalomyelitis (EAE), the mouse model of multiple sclerosis, that T cells reactive to the autoantigen myelin basic protein (MBP) localize to the sites of inflammation around the CNS [34]. This observation has been utilized in the delivery of anti-inflammatory cytokines to the CNS of EAE mice [35]. MBP-reactive T cells were infected with retroviral vectors encoding IL-4 ex vivo. Infected cells were sorted by FACS, and adoptively transferred into mice that had been immunized with MBP so as to induce EAE. The cells were found to localize to the CNS and to secrete IL-4 to sufficient levels as to delay the onset and severity of EAE. Despite the difficulties involved in

isolating and culturing such T cell lines, they make attractive delivery vehicles as they localize only to the sites of pathology, where they can secrete therapeutic proteins to high local concentrations.

A further possibility is to implant the retroviral packaging cells directly into the affected tissues to give a sustained supply of virus at a high local titer. Although some preliminary experiments have utilized this approach, it is probably not desirable in a therapeutic context given that some control of virus administration would be required. Having said that, it may be possible to incorporate a 'suicide' gene, such as thymidine kinase into the packaging cells. This would allow the packaging cells to be destroyed by administration of the appropriate prodrug (in the case of thymidine kinase this would be ganciclovir) to eliminate the source of virus once autoimmune pathology is under control.

Retroviruses and lentiviruses clearly have many attractive features as gene therapy vectors. How can these vectors be utilized in the treatment of autoimmune diseases? Several different strategies can be used: restoration of function lost as a result of autoimmunity, addition of effectors to interfere with cytokine function or signal transduction, and the addition of cytokines themselves. The latter two approaches can be used to either inhibit the destructive inflammatory response, or to induce tolerance to the autoantigen.

Restoration of Function

The current therapy for insulin-dependent diabetes mellitus requires daily injections of recombinant insulin, as the cells responsible for insulin production, beta cells found in the pancreatic islets, are destroyed by the autoimmune response. Although tens of thousands of people around the world manage to follow this regime, it is far from ideal. One solution would be to use gene therapy to deliver the proinsulin gene to the patient. This is not as straightforward as it first appears, as insulin production in the islet cells of healthy individuals is a tightly regulated process responsive to the level of blood glucose.

Hepatocytes are the target cell of choice in such strategies, as they constitutively express the pancreatic beta cell glucose transporter GLUT2 and the glycolytic enzyme glucokinase which are both required for the islet cells to monitor blood glucose levels. Repeated infection of primary rat hepatocytes in culture with a retrovirus expressing human proinsulin gave up to $40 \pm 10\%$ infection efficiency, resulting in 18.1 ± 7.9 ng of proinsulin per 24 h/ml of medium [36]. However, proinsulin production was not regulated, and hepatocytes do not possess the proteases necessary for processing proinsulin to give insulin. Chen et al. [37] placed the chloramphenicol acetyl transferase reporter gene

under the control of the glucose-dependent promoter of the L-type pyruvate kinase gene in an MMLV-derived vector. These viruses were used to infect cultured hepatocyte lines. In infected cells, the reporter showed the same responsiveness to glucose as did endogenous L-type pyruvate kinase.

A problem with both of these approaches is that in vivo in a healthy liver, hepatocytes have a low turnover rate, meaning that only a small proportion at a given time will be infectable by retroviruses. For such a therapy to succeed it would be better to use lentiviral vectors. Even if such a therapeutic strategy was to work, it fails to get to the root of the disease, the immune dysfunction which led to the destruction of the pancreatic islets. Manipulation of the immune response is the subject of the remainder of this review.

Cytokine Inhibitors

The classical view of T helper cells divides this group of lymphocytes into two groups, Th1 cells that are proinflammatory and mediate the cytotoxic response, and Th2 cells which mediate the humoral response. Although it is becoming increasingly apparent that this view is naive, it does serve as a basis for understanding the pathogenesis of autoimmunity. These groups are characterized by the cytokines they produce. Th1 cells produce TNF-α, IFN-γ and IL-2, whilst Th2 cells produce IL-4, IL-5 and IL-10. Both groups respond positively to autocrine stimulation, but inhibit each other. Therefore once an immune response has headed down either the Th1 or Th2 path, it is very difficult to stop. Many autoimmune diseases have been characterized as resulting from an inappropriate Th1 response leading to tissue damage. In vitro studies suggest that interfering with Th1 cytokines, or adding Th2 cytokines, can block autoimmune pathology.

Rheumatoid arthritis is a chronic inflammatory disease characterized by inflamed joints and cartilage destruction. Both TNF-α and IL-1 have been identified as key mediators in promoting disease. Successful use of anti-TNF-α monoclonal antibodies, soluble TNF-α receptors and a naturally occurring IL-1 receptor antagonist (IL-1Ra) protein in blocking the activities of these cytokines has opened up a new avenue of therapeutic possibilities [38]. The administration of proteins as drugs is problematic, given the difficulties of synthesis, purification, serum half-life and inactivation by the immune response, thus making gene therapy a viable strategy.

IL-1Ra is a small, acidic glycoprotein that blocks cellular responses to IL-1 by binding to the type I IL-1 receptor without triggering an agonistic response [39]. Initial work focused on implanting primary synoviocytes which had been infected ex vivo with a retrovirus encoding secreted (s)IL-1Ra into

the joints of rats and rabbits [40, 41]. Injection of recombinant IL-1β into the knees of rabbits leads to arthritis-like symptoms (leukocyte infiltration, synovial thickening, hyperplasia and loss of cartilage) [42]. These effects were inhibited in knees containing grafted modified cells, which produced nanogram quantities of sIL-1Ra. Can this same effect be seen in arthritic animals? Modified synoviocytes expressing sIL1-Ra were implanted into the knees of rats with bacterial cell-wall-induced arthritis [43]. Gene expression from the engrafted cells continued for at least 9 days after engraftment. Expression of sIL-1Ra suppressed the severity of the recurrence of arthritis in terms of joint swelling, and reduced, but did not completely abolish, bone and cartilage erosion. The effects of sIL-1Ra were confined to the sites of engraftment, with no significant difference in severity of arthritis in nongrafted joints between the control and experimental groups. This is a very encouraging finding, as one of the concerns surrounding cytokine therapies is the possibility of severe, system-wide side effects. It was estimated that locally expressed sIL-1Ra was approximately four orders of magnitude more efficient than systemically administered sIL-1Ra recombinant protein, presumably because of the high local concentration of sIL-1Ra around the synoviocyte grafts.

Although cell therapy is a plausible clinical approach, it would be preferable to be able to deliver the sIL-1Ra gene using a retrovirus in vivo. Initial studies attempted to compare direct injection of retrovirus into inflamed synovium with implantation of the retroviral packaging cell line. Using a retrovirus carrying lacZ and neomycin resistance markers, it was found that no cells were infected when retrovirus was directly injected into the joints, and only 1% of cells were infected in joints into which the packaging cells had been grafted [44]. These poor results were probably due to low viral titers and weak expression from the retroviral LTR. In later work using the MMLV-derived vector MFG-IRAP, Evans' group [45] has successfully delivered sIL-1Ra to the knee joints of rabbits. (The MFG vector exhibits high levels of transgene expression.) Initial experiments using human growth hormone as a secreted marker indicated that there was no difference in transduction efficiency when retroviral vector was administered either ex vivo or in vivo, with transgene expression lasting for at least 4 weeks. The highest transgene expression was found in arthritic, rather than nonarthritic, knees, with up to 20 ng sIL-1Ra per joint. This was to be expected, as cell proliferation is increased in arthritic joints, giving a greater percentage of the cell population that is susceptible to retroviral infection. A strong inhibition of matrix synthesis and an increase in cartilage catabolism was observed, though only a mild anti-inflammatory effect was seen. Although there was a reduction in leukocyte infiltration, this effect declined with time, and no other indices of inflammation were affected. It was interesting to observe that concentrations of rabbit IL-1 decreased with

time after expression of sIL-1Ra, suggesting the presence of an autocrine induction loop that may drive a runaway autoimmune response in arthritis. Similar results have also been observed in the collagen-induced arthritis (CIA) model in the mouse and in osteoarthritis in dogs [46]. One criticism of these experiments is that the transgene is administered before the onset of arthritis. Can these experimental therapies help if arthritis is already in progress? In vivo retroviral delivery giving sustained transgene expression should not be a problem in this scenario. Nguyen et al. [47] have shown that injection of very high titer retroviral vector (10^9 CFU) carrying the β-gal reporter gene into the ankles of rats with adjuvant arthritis gave β-gal expression peaking at 3–7 days after infection, and sustained at lower levels for at least 49 days. Injection of vector took place 15 days after administration of adjuvant. The success of this work may be due to the high rate of cell proliferation in the arthritic joint. From the point of view of safety it is reassuring to see that β-gal expression was restricted to the site of injection, and was not found in the contralateral uninjected paw, or in the spleen, lungs, kidneys or liver.

In light of these promising results using sIL-1Ra, similar strategies have been successful in inhibiting other cytokines. As mentioned earlier, another key mediator in the development and progression of rheumatoid arthritis is TNF-α. Anti-TNF-α monoclonal antibodies have been successfully used in human phase I and II clinical trials, alleviating the inflammation found in arthritic joints. Monoclonal antibodies are expensive to produce and purify. Given that retroviral vectors have been shown to be effective in delivering transgenes directly to the synovium, what are the options available for gene therapy strategies for interfering with TNF-α? One possible approach is to deliver soluble TNF-α receptor in the hope that this will be secreted across the synovium and mop up any TNF-α present before it can reach endogenous cellular receptors and trigger CTL and inflammation [48, 49]. Splenocytes were infected ex vivo with a retroviral vector carrying the cDNA for the soluble p75 TNF-α receptor [50]. The transduced cells were transferred to SCID mice into which CIA had been passively transferred with spleen B and T cells from DBA/1 mice. Mice carrying p75-transduced cells failed to develop arthritis. Analysis of IgG subclass levels and joint histology suggsted that a downregulation in Th1 may at least be partially responsible for the observed inhibition of disease. In contrast, if splenocytes were transduced with a retrovirus carrying the TNF-α cDNA, then the recipients showed exacerbated arthritis.

An alternative solution is to deliver antibodies expressed from the retroviral vector. Native immunoglobulin molecules are multi-molecular assemblies consisting of two heavy and two light chains. Obviously it is asking too much to expect both chains to be expressed in equimolar quantities and then be correctly assembled when delivered by a retroviral vector. An alternative is to

use an scFv derived from an anti-TNF-α monoclonal antibody. Preliminary data suggest that anti-TNF-α scFv can inhibit killing of tumor cells in an in vitro assay of TNF-α activity [R. Smith, unpubl. obs.], though further work is needed to improve the stability of the secreted molecule, possibly by addition of an immunoglobulin constant region domain. This would allow dimerization of the scFv polypeptides, which has been shown to greatly increase activity and stability.

Cytokine Therapy

It is clear that interfering with proinflammatory cytokines can be potentially useful as therapy for treating autoimmune disease. As described earlier, the immune response can crudely be considered to hinge on the balance between Th1 and Th2 cells. Rather than using inhibitory factors to block inflammation, could it be possible to deliver Th2 cytokines in order to stop the proinflammatory Th1 response.

It has already been described how IL-4 has been delivered to the CNS of EAE mice via retrovirally transduced autoreactive T cells, and has successfully blocked disease development [34, 35]. Similar success has also been found in the treatment of diabetes in the nonobese diabetic mouse model [51]. Pancreatic islet-specific Th1 clones were transduced with retroviral vectors encoding IL-10 under the control of the SRα promoter. Adoptive transfer of the transduced cells into nonobese diabetic mice led to the suppression of diabetes. This was correlated with a decrease in IFN-γ, measured at the level of mRNA, indicating that the delivery of Th2 cytokine was inhibiting, at least in part, the Th1 response (of which IFN-γ is an indicator).

Most research to date in the field of cytokine gene therapy has focused on trying to enhance immune responses against tumors, and has largely ignored autoimmunity. However, the successes seen in the prototype cancer therapies suggest that retrovirally delivered cytokine genes could be effective modulators of the immune response.

Induction of Tolerance

Although modifying the immune response with cytokines and cytokine inhibitors is taking a step in the right direction towards correcting the underlying immunological dysfunction of autoimmunity, this approach still does not get completely to the root of the problem. Is it possible to reeducate the immune system into becoming tolerant of autoantigens? How is tolerance

achieved? There are two basic mechanisms: T cell clonal deletion or clonal inactivation. Retroviral vectors have been used in 'brute force' approaches to eliminate superantigen or alloreactive T cells [52, 53] though it may be preferable to induce tolerance rather than risk a runaway elimination of multiple T cell lineages.

During development, deletion of self-reactive T cell clones occurs in the thymus. Delivering large quantities of autoantigen to the thymus by implanting tissue from the target organ can restore tolerance, though delivery of the antigen by a viral vector would obviously be preferable. However, is it possible to deliver a retroviral vector to the thymus? Marshall et al. [54], using the Gross murine leukemia virus (GMuLV), have managed to specifically infect medullary thymic epithelial cells. When this is done in neonatal mice, a complete abolition of GMuLV-specific cytolytic immune function was observed. It is interesting to note that the tissue specificity in this system is at the level of the U3 region of the viral LTR, rather than in the envelope.

The induction of tolerance in the adult has been of interest in the field of transplantation, though is clearly of great importance when considering autoimmunity. Tolerance to MBP in EAE mice can be induced by oral administration of MBP [55], implying that the route of delivery and dose of an antigen play a significant role in the immune response to that protein, as intravenous administration of MBP leads to development of EAE. This is probably due to different populations of antigen-presenting cells being involved in differnt routes of antigen delivery. Simply coculturing retroviral vectors encoding an autoantigen with bone marrow, then reintroducing the transduced bone marrow back into mice appears to induce tolerance. Ally et al. used the murine experimentally induced diabetes model for this work [56]. Transgenic mice expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein on the β-islet cells in the pancreas develop autoimmune diabetes when infected with LCMV. These animals were infused with bone marrow that had been transduced with an LCMV glycoprotein-encoding retrovirus, and were shown not to have LCMV-reactive T cell clones, unlike control animals. However, the precise cell populations that became infected were not characterized, so it cannot be said exactly how tolerance was induced. It may simply have arisen from large quantities of autoantigen being expressed, as levels of autoantigen do appear to play a role in determining whether an autoreactive T cell clone evades deletion or inactivation. If low levels of autoantigen are introduced, then tolerance is not induced, though it is unclear how this can be. If this were truly the case, then retroviral or lentiviral vectors would make attractive vehicles for expressing large quantities of autoantigen in antigen-presenting tissues.

These approaches are dependent on targeting subsets of antigen presenting cells that are capable of inducing tolerance. Is there any way in which to ensure that the targeted population of antigen-presenting cells induce tolerance? For dendritic cells to activate T cells, a number of costimulatory signals, in addition to contact between the T cell receptor and the major histocompatibility complex, are required. These signals are mediated by the binding of CD40 to CD154 and CD80/CD86 to CD28/CTLA4 (dendritic cell receptors listed first). Can these costimulatory signals be downregulated? Delivery of the cytomegalovirus cytokine homolog vIL-10 to dendritic cells in a retroviral vector has been used as a method of limiting the T cell activation potential of dendritic cells [57]. vIL-10 has high homology with native IL-10, but while vIL-10 has immunoinhibitory effects, it lacks the stimulatory activities of native IL-10. Dendritic cell progenitors were repeatedly transduced with the retroviral vector to give up to 40% infection efficiency. Transduced cells showed reduced surface expression of MHC class II and costimulatory molecules, leading to a reduced ability to stimulate T cells. Alternatively, it may be possible to block the costimulatory signals by expressing specific intracellular scFvs. scFvs derived from monoclonal antibodies against surface molecules have been used to trap the surface receptors inside the endoplasmic reticulum by virtue of a C-terminal KDEL tetrapeptide in the scFv, and effect phenotypic change of the target cell [58]. This signal peptide marks the scFv-receptor complex for entrapment in the endoplasmic reticulum. Such constructs have been delivered to CD80+ B cells by retroviral infection, and have been observed to reduce surface CD80 expression, though it is as yet unclear whether this will be sufficient to induce tolerance [R. Smith, unpubl. obs.].

Future Perspectives

Retroviruses are clearly attractive choices as vectors for efficiently delivering transgenes in many situations. However, as lentiviral vector technology matures it is likely that these vectors will eventually replace traditional retroviruses. Many of the applications described in this review would benefit from the ability of lentiviruses to infect nonproliferating cells, in particular hematopoietic stem cells and dendritic cells. Although the nature of autoimmunity and the induction of tolerance still have many unanswered questions, the model systems described here should hopefully allow the interactions and activities of the various molecules involved in antigen presentation and T cell activation to be elucidated in addition to providing new therapies for autoimmune disease.

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