Transgenic Mice as Immunogenicity Models

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Introduction

Antibody development after administration of native or recombinant produced pharmaceutical proteins has been reported for many proteins, e.g., insulin, human growth hormone (hGH), coagulation Factor VIII and Factor IX (*Reeves 1983 and 1985 and 1986; Okada et al. 1987; Kaplan et al. 1986; Schernthaner et al. 1983; Roberts & Cromatie 1984*). The formation of insulin antibodies was reported shortly after the introduction of insulin therapy. Impurities were the main reason for antibody formation, but even with the highly purified insulin's now available insulin antibodies are still detected in patients *(Kasama et al. 1981; Root et al. 1972; Schlichtkrull et al. 1972; Van Haeften 1989)*. Insulin antibodies have been reported to play a role in insulin allergy, the development of injection site lipoatrophy, insulin resistance and poor glycemic control due to altered insulin pharmacokinetics (*Reeves 1983; Van Haeften 1989*). Antibodies against hGH have been reported to cause growth attenuation (*Okada et al. 1987; Kaplan et al. 1986*). Antibodies against FVIII and FIX have resulted in FVIII/FIX resistance with prolonged bleeding time (*Roberts & Cromatie 1984*).

It must be a requirement that efforts are made to evaluate the potential immunogenicity of a new pharmaceutical protein before the protein is administered to patients in clinical trials.

It is desirable to have a model where the potential immunogenicity of new pharmaceutical proteins can be evaluated in order, to ensure that a protein to be used in clinical trials has been tested in the best possible way.

Transgenic mice have been used extensively as a way to elucidate how the immune system is capable of distinguishing between self and non-self and several comprehensive reviews are available (*Adams 1990; Miller et al. 1991; Lo et al. 1990 and 1991; Basten et al. 1991*)

In this review results obtained using transgenic mice to evaluate the potential immunogenicity of recombinant proteins and the models advantages and drawbacks will be discussed.

Traditional Immunogenicity Models

One approach to test the potential immunogenicity of pharmaceutical proteins has been to use newborn mice that are artificially made tolerant by single or repeated injection of the native protein, followed by subsequent challenge with the protein that one wishes to test the immunogenicity of (*Nossal 1989; Siskind 1984*). The newborn mouse model has the disadvantage that the tolerance established has little resemblance to the tolerance seen in animals that have tissue specific expression of physiological levels of the protein from early stages of embryonic development (*Lipes & Eisenbarth 1990*).

The cellular immune response to insulin has been evaluated in different *in vitro* systems using peripheral blood lymphocytes from humans to assay for T cell proliferation (*Parkar & Reeves 1989; Nell & Thomas 1983*). Since there is considerably heterogeneity in the lymphocyte responses in human individuals, it has been difficult to standardize these assays.

In the absence of more appropriate models, laboratory animals (e.g., mice, rats, guinea pigs, rabbits and primates), in which the endogenous protein do not deviate to much from the protein to be tested, has been used to evaluate the immunogenicity of pharmaceutical proteins before administration to patients. Since rabbit insulin only differs from human and porcine insulin at one amino acid residue (*Schlichtkrull et al.1974*), a

rabbit model has been used to evaluate the influence of varying levels of impurities in early insulin preparations (*Root et al. 1972; Schlichtkrull et al. 1972 and 1974*).
Finally, although it cannot be classified as an immunogenicity model, the only way to discover the immunogenicity of pharmaceutical proteins has sometimes been retrospective evaluation in patients.

Transgenic Mice as Immunogenicity Models
The idea of using transgenic mice as immunogenicity models has been suggested based on the hypotheses, that a mouse transgenic with a human gene, is not expected to produce antibodies against the human protein that the gene is coding for, because the immune system will recognize the protein as self (*Stewart et al. 1989; Mikkelsen et al. 1992; Ottesen et al. 1994*).
The main advantage of using transgenic mice is that tolerance to the native protein has been induced from early embryonic development. In contrast to the traditional laboratory animals, the transgenic immunogenicity model includes a positive control in the non-transgenic littermates, whereas the only positive control in conventional laboratory animals consists of known immunogenic preparations. Different mouse strains must be carefully evaluted before the model is used, because the MHC haplotype in itself may cause tolerance to a given protein in the non-transgenic mice, which should serve as a positive control.

Human insulin

Insulin has been used for many years for basic immunological research. The main reasons to use insulin have been that it is available in highly purified form; the primary and tertiary structure has been known for many years; several useful analogues have been prepared and the fact that insulin is highly conserved among species, e.g., human, porcine, bovine, horse and rabbit insulin differ by only 1-3 amino acids (*Keck 1975 and 1981; Reeves 1983*).

The insulin preparations used for treatment of diabetic patients cannot mimic the normal physiological rapid increment of the insulin level in the blood at the time of meal consumption, probably because the insulins are mostly assembled as zinc-containing hexamers, which may limit the rate of absorption from the injection site. DNA technology has made it possible to make human insulin analogues with amino acid substitutions or deletions designed to prevent hexamer formation. This was done by introducing amino acids that results in charge repulsion in the monomer-monomer interface without destabilizing its own three-dimensional structure or interfering with its biological activity (*Brange et al. 1988 and 1990*).
No matter how great *in vivo* advantages the analogues possess, there will always be the possibility that an immunogenic epitope has been created. Antibodies directed against such immunogenic epitopes might cross react with the native protein and have a neutralizing effect on both the analogue and the endogenous protein and thereby aggrevate the patient's condition.
In a recent paper *Ottesen et al.* (1994) use human insulin transgenic mice and non-transgenic control mice (H-$2^{d/b}$) immunized with human, porcine, bovine and rat insulin and 12 human insulin analogues with substitutions/deletions at 1-3 amino acid residues, to evaluate the potential immunogenicity.
Bovine insulin differs from human insulin (Fig. 1) at position 8 and 10 in the A-chain and position 30 in the B-chain. Porcine insulin differs from bovine insulin at residue B30. Rat insulin was used to show that the mouse immune system is self-tolerant since rat and mouse insulin are identical. Sera from the immunized mice were assayed in radio immuno assay (RIA) and ELISA with similar results. The RIA results (Fig. 2) show that tolerant transgenic mice develop antibodies against bovine insulin and human insulin analogues with amino acid substitution at position 8 and 10 in the A-chain loop. The results with human, porcine and bovine insulin

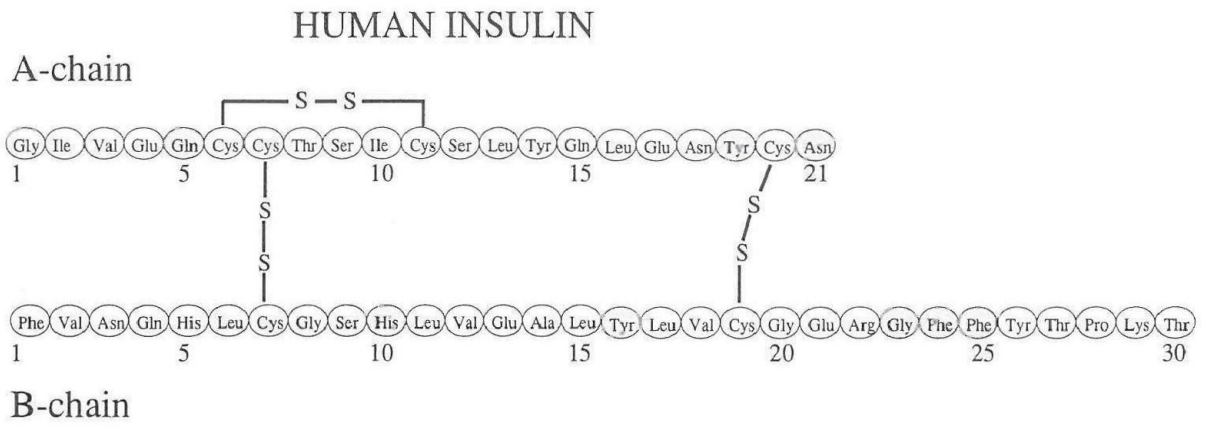

Fig. 1. Primary structure of human insulin. Putative sites interacting with the insulin receptor are indicated with grey residues (*Brange et al. 1990*). The analogues shown in Fig. 2 are named after the positions in the A and B chain where the substitutions or deletions have been made; e.g., in the analogue A21G/B3D/B27- the amino acids asparagine (N) in human insulin at position A21 and B3 have been substituted with the amino acids glycin (G) and aspartate (D), and threonine (T) at position B27 have been deleted. Amino acid differences between human insulin and animal insulin: Bovine (A8A/A10V/B30A); Porcine (B30A); Mouse/Rat I (A4D/B3K/B9P/B30S); Mouse/Rat II (A4D/B3K/B29M/B30S).

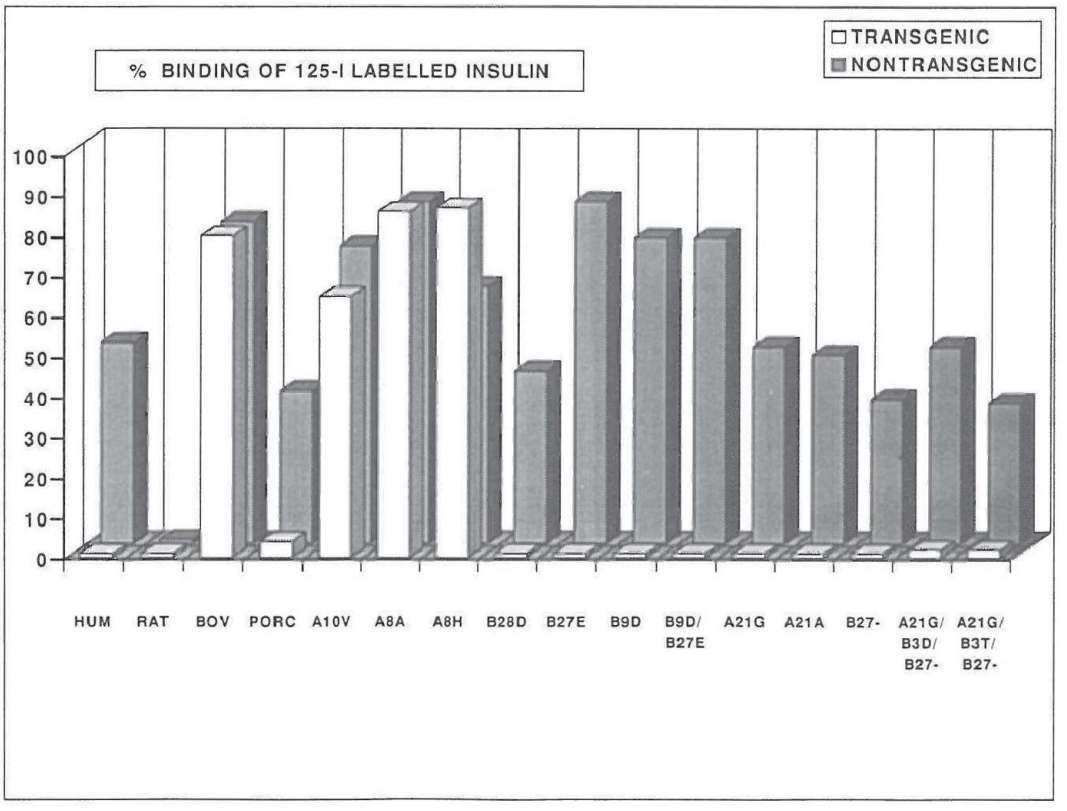

Fig. 2. Antibody responses to insulins and insulin analogues in transgenic mice and non-transgenic controls (*Ottesen et al. 1994*). RIA data are presented as the groups mean %-binding of ^{125}I labelled insulin/analogue (9-16 mice in each group). Standard error of mean was 0.1-7.0 for transgenic groups and 0.1-12.8 for non-transgenic groups. Except for rat insulin, bovine insulin and the analogues A8A, A8H and A10V there are significant difference between transgenic and non-transgenic groups ($p<0.03$).

reflect the clinical situation (*Jensen & Kapp 1984; Schernthaner et al.,1983)*. Keck have shown that the bovine epitope in the A-chain loop (A8-A10) elicit an antibody response in responder mice (*Keck 1975 and 1977*), and the antibody response in transgenic mice against the 3 analogues with single amino acid substitutions in the A-chain loop gives the first *in vivo* indication, that only one substitution in the A-chain loop is enough to elicit an antibody response.
It is further demonstrated that the antibodies developed against the various insulins are extremely cross reactive against human insulin. The non-transgenic control mice developed antibodies against all insulins and analogues except against rat insulin (*Ottesen et al. 1994*).

Human tissue plasminogen activator (htPA)

Stewart et al. (1989) has used transgenic mice, that express human tissue plasminogen activator (htPA) to which the mice are shown to be immunologically tolerant, as a model to evaluate whether an immune response could be produced against an analogue in which a single amino acid had been altered. The transgenic mice and their non-transgenic siblings were injected with htPA and a htPA analogue where arginine at position 275 were replaced by glutamic acid (htPA-E275).
None of 21 transgenic mice developed htPA antibodies when injected with htPA in contrast to the non-transgenic controls where 40/42 developed htPA antibodies. When injected with the htPA-E275 analogue 8/44 transgenic mice and 79/80 non-transgenic mice developed antibodies against this analogue. It was further shown that the antibodies developed in the 8 transgenic mice against htPA-E275 cross-reacted with htPA. In a parallel setup *Stewart et al.* showed that when transgenic and non-transgenic mice where immunized using Freunds adjuvant with htPA or the analogue all the control mice developed antibodies against both htPA and the analogue, whereas 50% of the transgenic mice produced antibodies against the analogue, but the tolerance against htPA was maintained in transgenic mice immunized with htPA.

Human growth hormone (hGH)

Martin et al. (1993) reports that changes in tertiary structure of hGH can be detected in hGH transgenic mice. These mice makes no detectable antibodies following challenge with monomeric hGH, but produce anti-hGH antibodies after immunization with misfolded hGH. This suggests that tolerant transgenic mice could be used as a model to ensure similarity between recombinantly produced protein and the native protein. Non-transgenic mice will develop antibodies against both the pharmaceutical protein and possible contaminants, whereas an antibody response in tolerant transgenic mice (determined using ELISA plates coated with the preparation used for immunization), are raised only against misfolded protein or against contaminants. Verification can be obtained in a western blot. If monoclonal antibodies can be produced by the transgenic mouse strain, these antibodies can be used for specific removal of the contaminants.

Future Transgenic Immunogenicity Models

Other models may be developed which aim at making the mouse response more »human«. Research with immunodeficient SCID mice, which have neither functional T cells nor B cells, has demonstrated that these immunodeficient mice allows reconstitution with neonatal elements from the human immune system, i.e., fetal liver, bone marrow, thymus and peripheral lymphoid organs such as lymph node, spleen and skin, without the development of graft-versus-host symptoms (*Kaneshima et al. 1990; McCune 1991*). SCID mice engrafted with human hematolymphoid organs (SCID-hu) have been immunized with test antigens and been found to generate specific human antibodies against these antigens (*Kaneshima et al. 1990*). If further research with the SCID-hu mice proves that these mice can mimic the human immune sy-

stem, it should be possible to produce a transgenic SCID-hu mouse that expresses human insulin and use them as described in this paper. Such transgenic SCID-hu mice should, in theory, react with a human immune response. Future experiments will show whether this theory will prove to be valid.

In mice transgenic with human insulin only 4 amino acid residues differ between mouse and human insulin, but in other trangenic models there will be less similarity between the amino acid sequence of the mouse protein and the protein coded by the transgene. If human analogues are developed where substitutions resemble epitopes on the endogenous mouse protein, the mouse immune system will recognize the epitopes as self, and no antibody response will be elicited. If the gene sequence of both the mouse and the human gene is known, this problem could be overcome by knock out of the endogenous mouse gene by gene targeting in embryonic stem cells (*Capecchi 1989; Sedivy & Joyner 1992*). Using this approach one could for example make a transgenic mouse that produce human insulin but not mouse insulin.

It is important to emphasize that, as with all other models with the aim of predicting the potential immunogenicity of pharmaceutical proteins, the transgenic immunogenicity model should be carefully validated before it is used. Special attention has to be taken to the MHC haplotype of the mice used, the immunization protocol and the detection assays.

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Summary
The development of antibodies after administration of pharmaceutical proteins is not only of academic interest. Antibodies can be responsible for local and systemic allergic reactions, injection site lipoatrophy and have effect on the dose requirements. Tolerant transgenic mice are used to evaluate the potential immunogenicity of pharmaceutical proteins, e.g., human insulin, human tissue plasminogen activator and human growth hormone. The results indicate that transgenic mice should be useful as an in vivo model to map immunogenic epitopes. Transgenic mice with tissue specific expression of human insulin that are tolerant to human insulin (in contrast to their non-transgenic littermates that produce antibodies) are able to respond with antibody formation against human insulin with substitution of single amino acids, if the substitutions results in immunogenic epitopes.
Finally potential future immunogenicity models are discussed.

Sammendrag
Dannelse af antistoffer efter indgift af pharmaceutiske proteiner har ikke kun akademisk interesse. Antistoffer kan resultere i lokale og systemiske allergiske reaktioner, lipo-atrofi, og have en effekt på dosisbehovet. Tolerante transgene mus benyttes til at vurdere den potentielle immunogenicitet af pharmaceutiske proteiner, f.eks. human insulin, human tissue plasminogen activator og human vækst hormon. Resultaterne indikerer, at transgene mus kan benyttes som en *in vivo* model til at mappe immunogene epitoper. Transgene mus med vævsspecifik ekspression af human insulin, der er tolerante overfor human insulin (imodsætning til deres non-transgene kuldsøskende der danner antistoffer), danner antistoffer mod human insulin med substitution af en enkelt aminosyre, hvis substitutionen har resulteret i en immunogen epitop.
Potentielle fremtidige immunogenicitetsmodeller diskuteres.

References
Adams T.E.: Tolerance to Self-antigens in Transgenic Mice. *Mol Biol Med* 1990; 7: 341-357.
Basten A., R. Brink, P. Peake, E. Adams, J. Crosbie, S. Hartley, and C.C. Goodnow: Self Tolerance in the B-Cell Repertoire. *Immunol Rev* 1991; 122: 5-19.
Brange J., D.R. Owens, S. Kang, and A. Vølund: Monomeric Insulins and Their Experimental and Clinical Implications. *Diabetes Care* 1990; 13: 923-954.
Brange J., U. Ribel, J.F. Hansen, G. Dodson, M.T. Hansen, S. Havelund, S.G. Melberg, F. Norris, K. Norris, L. Snel, A.R. Sørensen, and H.O. Voigt: Monomeric insulins obtained by protein engineering and their medical implications. *Nature* 1988; 333: 679-682.
Capecchi M.R.: Altering the Genome by Homologous Recombination. *Science* 1989; 244: 1288-1292.
Jensen P.E. & J.A. Kapp: Regulatory Mechanisms of the Immune Response to Heterologous Insulins. I. Development and Regulaion of PFC Responses in Vitro. *Cell Immunol* 1984; 87: 73-84.

Kaneshima H., C. Baum, B. Chen, R. Namikawa, H. Outzen, L. Rabin, A. Tsukamoto, and J.M. McCune: Today's SCID-hu mouse. *Nature* 1990; 348: 561-562.

Kaplan S.L., L.E. Underwood, G.P. August, J.J. Bell, S.L. Blethen, R.M. Blizzard, D.R. Brown, T.P. Foley, R.L. Hintz, N.J. Hopwood, A. Johansen, R.T. Kirkland, L.P. Plotnick, R.G. Rosenfeld, and J.J. Van Wyk: Clinical studies with recombinant-DNA-derived methionyl human growth hormone in growth hormone deficient children. *Lancet* 1986; 1: 697-700.

Kasama T., Y. Iwata, K. Oshiro, M. Uchida, Y. Sakaguchi, K. Namie, and M. Sugiura: Antigenicity of Desamido-Insulin and Monocomponent Insulin. *Diabetologia* 1981; 21: 65-69.

Keck K.: Ir-gene control of immunogenicity of insulin and A-chain loop as a carrier determinant. *Nature (Lond)* 1975; 254: 78-79.

Keck K.: Ir gene control of carrier recognition. III. Cooperative recognititon of two or more carrier determinants of different species. *Eur J Immunol* 1977; 7: 811-816.

Keck K.: Insulin as a tool for the study of immunological problems. In: Keck K. and P. Erb, eds. *Basic and clinical aspects of immunity to insulin.* Berlin, New York: Walther de Gruyter & Co.; 1981:3-15.

Lipes M.A. & G.S. Eisenbarth: Transgenic Mouse Models of Type 1 *Diabetes.* Diabetes 1990; 39: 879-884.

Lo D., L.C. Burkley, R.A. Flavell, R.D. Palmiter, and R.L. Brinster: Antigen Presentation in MHC Class II Transgenic Mice: Stimulation versus Tolerization. *Immunol Rev* 1990; 117: 121-134.

Lo D., J. Freedman, S. Hesse, R.L. Brinster, and L. Sherman: Peripheral Tolerance in Transgenic Mice: Tolerance to Class II MHC and non-MHC Transgene Antigens. *Immunol Rev* 1991; 122: 87-102.

Martin L., M. Mora-Worms, C. Lucas, C. Reynolds, and T.A. Stewart: An evaluation of mechanisms by which tolerance to organ-specific antigens is lost using a transgenic mouse model. *J Immuol* 1993; 150: 1234-1243.

McCune J.M.: SCID mice as immune system models. *Current Opinion in Immunology* 1991; 3: 224-228.

Mikkelsen T.R, B. Chapman, N. Din, J. Ingerslev, P. Kristensen, K. Poulsen, and J.P. Hjort: Expression of a cytomegalovirus IE-1-factor VIII cDNA hybrid gene in transgenic mice. *Transgenic Research* 1992; 1: 164-169.

Miller J.F.A.P., G. Morahan, J. Allison, and M. Hoffmann: A Transgenic Approach to the Study of Peripheral T-Cell Tolerance. *Immunol Rev* 1991; 122: 103-116.

Nell L.J. & J.W. Thomas: The human immune response to insulin. Kinetic and cellular aspects of lymphocyte proliferative responses in diabetics. *J Immunol* 1983; 131: 701-705.

Nossal G.J.V.: Immunologic Tolerance: Collaboration Between Antigen and Lymphokines. *Science* 1989; 245: 147-153.

Okada Y., K. Taira, K. Takano, and N. Hizuka: A case report of growth attenuation during methionyl human growth hormone treatment. *Endocrinologia Japonica* 1987; 34: 621-626.

Ottesen J.L., P. Nilsson, J. Jami, D. Weilguny, M. Dührkop, D. Bucchini, S. Havelund, and J.M. Fogh: The potential immunogenicity of human insulin and insulin analogues evaluated in a transgenic mouse model. *Diabetologia* 1994; 37: 1178-1185.

Parkar B.A. & W.G. Reeves: In vitro priming of human lymphocytes to heterologous insulins. *J Immunol Meth* 1989; 120: 159-165.

Reeves W.G.: Insulin Antibody Determination: Theoretical and Practical Considerations. *Diabetologia* 1983; 24: 399-403.

Reeves W.G.: Immunogenicity of insulin of various origins. *Neth J Med* 1985; 28: 43-46.

Reeves W.G.: The immune response to insulin: Characterisation and clinical consequences. In: Alberti KGMM, Krall LP, eds. *The Diabetes Annual Vol 2.* Amsterdam: Elsevier Science Publishers; 1986:81-93.

Roberts HR & R. Cromatie R: Overview of Inhibitors to Factor VIII and IX. In: Hoyer LW, ed. *Factor VIII Inhibitors.* New York: Alan R. Liss; 1984:1-18.

Root M.A., R.E. Chance, and J.A. Galloway: Immunogenicity of Insulin. *Diabetes* 1972; 21 (Suppl. 2): 657-660.

Schernthaner G., M. Borkenstein, M. Fink, W.R. Mayr, J. Menzel, and E. Schober: Immunogenicity of Human Insulin (Novo) or Pork Monocomponent Insulin in HLA-DR-Typed Insulin-dependent Diabetic Individuals. *Diabetes Care* 1983; 6: 43-48.

Schlichtkrull J., J. Brange, A.H. Christiansen, O. Hallund, L.G. Heding, and K.H. Jørgensen: Clinical Aspects of Insulin-Antigenicity. *Diabetes* 1972; 21 (Suppl. 2): 649-656.

Schlichtkrull J., J. Brange, A.H. Christiansen, O. Hallund, L.G. Heding, K.H. Jørgensen, S. Munkgaard Rasmussen, E. Sørensen, and A. Vølund: Monocomponent insulin and its clinical implications. *Hormone Metabolism Research* 1974; 5 (Suppl.): 134-143.

Sedivy J.M. & A.L.Joyner (Eds). Gene Targeting. New York: WH Freeman and Company; 1992:143-168.

Siskind G.W.: Immunological Tolerance. In: Paul WE, ed. *Fundamental Immunology.* New York: Raven Press; 1984:537-558.

Stewart T.A., P.G. Hollingshead, S.L. Pitts, R. Chang, L.E. Martin, and H. Oakley: Transgenic Mice as a Model to Test the Immunogenicity of Proteins Altered by Site-specific Mutagenesis. *Mol Biol Med* 1989; 6: 275-281.

Van Haeften T.W.: Clinical significance of insulin antibodies in insulin-treated diabetic patients. *Diabetes Care* 1989; 12: 641-648.