6
Foreign Peptides Expressed in Engineered Chimeric Self Molecules

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Introduction

Antigens are macromolecules that interact with specific lymphocyte receptors: B-cell immunoglobulin receptor (IgR) and T-cell antigen receptor (TCR). The antigen is envisioned as a macromolecule. However, the specific lymphocyte receptor does not see the whole antigen as a macromolecular unit, but rather as a restricted segment of it. The segment of the macromolecule recognized or that interacts with TCR or IgR is called antigenic determinant or epitope.

A single antigenic molecule often displays more than an antigenic determinant. In the case of proteins, the epitopes may correspond to primary or higher order macromolecular structures.

A category of epitopes interacting with IgR formed by molecular segments that are distant regarding the primary structure but are juxtaposed in the secondary and tertiary structure, are called conformational epitopes. They can be recognized by IgR only on the native molecules but never on denatured molecules.

A second category of epitopes is entirely determined by the primary structure of a segment of the antigen molecule, and are called sequential epitopes. Sequential epitopes are recognized by TCR only in association with MHC molecules (Zinkernagel and Doherty, 1974) or CD1 molecules (Castano *et al.*, 1995; Sieling *et al.*, 1995). Generally, T-cells recognize linear epitopes derived from the processing of proteins (Townsend *et al.*, 1986) but they also can recognize glycopeptides (Harding *et al.*, 1993). Sequential epitopes derived from the processing of proteins are recognized by T cells in association with MHC class I or class II molecules (data reviewed by Braciale *et al.*, 1992).

The advances of peptide chemistry made possible the preparation of synthetic peptides. This provided a new tool for immunological studies, leading to the structural

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Biotechnology and Genetic Engineering Reviews – Vol. 15, April 1998
0264–8725/98/15/159–198 $20.00 + $0.00

characterization of the epitopes and particularly, the components (i.e. amino acids, sugars, sugar linkages, etc.) that are critical for the interaction with IgRs or with TCRs.

The study of the immunogenicity of the peptides lead to the concept of immunodominant epitopes since it was established that among the multitude of peptides derived from an antigen, some are more immunogenic than the others.

The preparation of synthetic peptides opened new avenues of research and applications such as the development of new generations of peptide-based vaccines devoid of side effects, the characterization of pathogenic self-peptides responsible for the occurrence of autoimmune diseases as well as their antagonists that can be used as immunotherapeutic agents against these diseases.

While *in vitro* studies utilizing peptides allowed a substantial progress in our knowledge regarding the characterization of the critical structures interacting with various cell receptors, *in vivo* studies have seen a major drawback related to their short half-life. In addition to their short half-life, peptide-based vaccines are poorly immunogenic. The molecular weight of a substance is thought to determine the immunogenicity. As an empirical rule, molecules of higher molecular weight are more immunogenic. With few exceptions such as glucagon (2.5 kDa) which is immunogenic, the vast majority of polypeptides with a molecular weight less than 5 kDa are not immunogenic.

To increase the immunogenicity of peptides, several approaches have been undertaken. First, the administration of peptides in adjuvants or other delivery systems such as liposomes or ISCOMs. Secondly, the coupling of peptides, like in the case of haptens, to highly immogenic carriers (i.e. a haemocyanine, bacterial toxins or toxoids) or to ligands for various cell receptors: IgR, i.e. anti-Ig-peptide, class I or class II, i.e. anti-MHC antibody–peptide conjugates (Casten *et al.*, 1988); transferin receptor, i.e. transferin–peptide conjugate (McCoy *et al.*, 1993); α macroglobulin receptor, i.e. α macroglobulin–peptide conjugate (Mitsuda *et al.*, 1995); RF receptor (Roosnek and Lanzavecchia, 1991) or FcγRII (Liu *et al.*, 1996), i.e. multimeric immune complexes. Thirdly, the preparation of chimeric molecules expressing foreign peptides using molecular engineering techniques that enabled the insertion of oligonucleotides encoding T- or B-cell epitopes within the coding region of genes specifying unrelated proteins. T-cell epitopes were expressed in *E. coli* alkaline phosphatase (Freimerth and Steinman, 1990), Pho E protein (Janssen and Thomassen, 1994), Mal E, Lam (Leclerc *et al.*, 1989), flagellin (Newton *et al.*, 1989) or fimbriae of Bacteroides nodosum (Jennings *et al.*, 1980). Transfectant viruses expressing minigenes encoding unrelated microbial peptides recognized by T- or B-cells also lead to generation of chimeric molecules (Leclerc *et al.*, 1990; Clarke *et al.*, 1987; Rutgers *et al.*, 1988; Dedieu *et al.*, 1992; Li *et al.*, 1992; Hahn *et al.*, 1992).

This review is aimed at presenting the information related to the methodology of the utilization of self molecules as platforms for presentation of either foreign or self peptides. In addition, we will present the seminal information related to the ability of these molecules to elicit an immune response as well as the molecular mechanisms responsible for the recognition of peptides by lymphocytes.

Genetically engineered self molecules expressing foreign peptides

Ig molecules were generally used as 'self' platforms to express foreign epitopes for several reasons. First, studies derived from the internal image concept demonstrated

that anti-Id antibodies which share sequences with foreign antigens such as glutamic acid–alanine–tyrosine terpolymer (GAT) (Roth *et al.*, 1985) or reovirus's haemagglutinin (Bruck *et al.*, 1986), are able to stimulate an immune response by functioning as antigen surrogates (Bailey *et al.*, 1989; Gaulton *et al.*, 1986). X-ray crystal analysis showed that the majority of amino acid residues in the hypervariable region of antibody molecules that interact with antigen, also interact with internal image-anti-Id antibodies (Fields *et al.*, 1995).

Secondly, the half-life of foreign peptides can be considerably prolonged by virtue of the long half-life of IgG molecules.

Thirdly, the structure of IgG molecules was investigated in detail and the segments used to insert foreign epitopes exposed at surface were determined with accuracy.

Generally, the CDRs of VH or VL regions were used to insert the foreign epitopes because they provided a molecular scaffold to install foreign sequences without altering the conformation of CDR and permitting the exposure of the inserted peptides. The principle of the expression of foreign sequences in CDRs of VH or VL chains of Ig consists either in creation of a unique cloning site by site-directed mutagenesis which allows the insertion of an oligonucleotide coding for foreign epitopes (Sollazo *et al.*, 1990) or deletion of the existing CDR sequence and the replacement with a foreign sequence by PCR mutagenesis (Zaghouani *et al.*, 1992). The chimeric V-gene is then subcloned upstream from a constant region (Cγ or Ck) expression vector.

CHIMERIC Ig EXPRESSING LIGAND-SHORT PEPTIDES

The RGD peptide is expressed in various extracellular matrix proteins such as fibronectin, tenascin, or in microfibril's proteins such as fibrillin-1. The peptide binds to integrin cell surface receptors ($\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$) contributing to the anchoring of cells to the matrix proteins. Lee *et al.* (1993) inserted the RGD sequence into CDR of VL gene of RE-I protein. Unfolded or proteolitically degraded chimeric VL fragment expressing RGD tripeptide competed in the binding of various proteins such as fibrinogen, fibronectin, von Willebrand's factor and vitronectin to integrin cell receptors, namely the thrombocyte receptor $\alpha_{IIb}\beta_3$. It is noteworthy that the intact chimeric molecule did not compete in this assay, suggesting that RGD inserted into a CDR loop should be surface-exposed in order to interact with receptor molecules. Barbas *et al.* (1993), by using a semisynthetic combinatorial library, selected a human Fab expressing the RGD sequence in the CD3. This protein expressing the RGD tripeptide flanked by various amino acids, was able to compete with the binding of vitronectin and fibrinogen to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrin receptors. The affinity constant of the RGD peptide expressed in this construct was estimated to be 10^{-10} M.

Billetta *et al.* (1997) inserted one or three RGD sequences into the CDR3 of VH region cloned from a hybridoma producing antithyroglobulin autoantibodies. The chimeric Ig molecules expressing RGD or RGD_3 were able to bind to integrin receptors borne by tumor cells and to compete with vitronectin and fibrinogen for the binding to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrin receptors.

In the aggregate, these data indicate that surface exposure of RGD tripeptide naturally expressed into a CDR of Fab fragment of Ig molecule or genetically inserted in the CDRs of VL or VH gene can bind to integrin cell receptors.

CHIMERIC Ig EXPRESSING B PEPTIDES RECOGNIZED BY IgR (B CELLS)

Because antibodies interact with native antigen macromolecules in solution, the epitopes recognized by IgR should be predominantly surface exposed. This means that these epitopes are composed mainly of hydrophilic residues having electronegative atoms able to form hydrogen bonds or dipole interactions and are electrically charged. The segments made up of hydrophobic residues are hidden or buried in the three-dimensional structure and do not interact with IgR. Knowledge of structural and thermodynamic characteristics of B-cell epitopes implies that the major constraint on the expression of B-epitopes interacting with IgR consist in their surface exposure from the entire body of a macromolecule.

The CDR3 of the VH region of Ig molecules represented the best choice for the expression of B cell epitopes (Bona *et al.*, 1997) because:

(i) the CDR3 sequences are surface exposed;
(ii) the CDR3 fragments are strongly immunogenic and able to elicit an antibody response not only across xenogenic and allogenic but also in autologous systems;
(iii) the CDR3s vary in length and therefore permit the insertion of peptides of various sizes without altering the folding of the molecule (Bona *et al.*, 1994).

Solazzo *et al.* (1990) first reported the preparation of a chimeric Ig molecule expressing a peptide corresponding to the tandem repeat epitope of the circumsporozoite protein (CSP) of *Plasmodium falciparum.* The oligonucleotide encoding the peptide was inserted in the CDR3 of the VH gene of a hybridoma producing an autoantibody. Injected in rabbits, this chimeric Ig was able to induce the production of anti-CSP that prevented the infection of target cells with sporozoites (Billetta *et al.*, 1991). It is unknown whether the insertion of CSP peptide into the CDR3 of the VH segment preserved or abolished the self-reactivity, namely the binding to thyroglobulin of the parental antibody.

Zaghouani *et al.* (1995) chose a different strategy to express into a foreign Ig a B cell epitope. In contradistinction to Solazzo's approach of inserting into the CDR3 of the VH gene an oligonucleotide encoding a CSP peptide, Zaghouani *et al.* replaced the CDR3 by PCR mutagenesis, with an oligonucleotide encoding a HIV-1 gp120 peptide recognized by B cells (Zaghouani *et al.*, 1995) or the CDR2 with an oligonucleotide encoding an influenza virus haemagglutinin peptide (Brumeanu *et al.*, 1996a). The chimeric Ig expressing a 19-amino acid consensus sequence predicted from the sequencing of 245 HIV-1 isolates was also humanized by replacing the murine genes encoding Cγ2b and Ck with human Cγ1 and Ck (*Figure 1*).

The data in *Table 1* summarize the antigenic and immunogenic properties of the chimeric murine-human Ig molecules expressing the V3 loop consensus sequence called IgV_3C. The injection of baboons with IgV_3C induced neutralizing antibodies. Moreover, IgV_3C was able to stimulate *in vitro* the synthesis of anti-V_3C peptide antibodies by lymphocytes of HIV-1 positive, asymptomatic patients. It is noteworthy that the synthesis of anti-V_3C peptide antibodies was higher when the lymphocytes were exposed to IgV_3C coupled with tetanus toxoid. This suggested that tetanus toxoid-specific memory T-cells provided help to V_3C peptide-specific B cells.

Collectively, these results demonstrate that foreign epitopes expressed in the CDR3 of VH fragment of the Ig molecule are recognized by IgR of B-cells and are able to elicit an immune response.

Table 1. Antigenicity and immunogenicity of murine-human chimeric Ig expressing the V_3C peptide of HIV-1

Antigenicity

	Binding to antibodies specific for Human Vk	HIV-1 V_3C peptide
Human Ig	+	–
IgV_3C	+	+

Immunogenicity: properties of antibodies produced by baboons immunized with chimeric mouse–human Ig expressing or not V_3C peptide

Immunogen	Binding to HIV-1 V3C peptid-BSA conjugate (RIA)	Binding to HIV-1 MN lysate (Western)	Neutralization of MN isolate (MTT assay)
IgV_3C	+	gp 120	+ (1:200 serum dilution)
Wild Ig	–	–	–

CHIMERIC Ig MOLECULES EXPRESSING PEPTIDES RECOGNIZED BY CYTOLYTIC $CD8^+$ T CELLS

$CD8^+$ T cells mediate cytotoxic immune responses upon recognition of peptides in association with MHC class I molecules (Bastin *et al.*, 1987).

Basically MHC class I molecules present to T-cells the peptides derived from the endogenous pathway of protein processing. The proteins devoid of signal sequences are processed within cytoplasm, whereas those bearing signal sequences are processed within the ER compartment. In cytoplasm, the antigen processing occurs in the proteasomes, which is a major extralysosomal proteolytic system. A cytosolic protein that undergoes processing is first associated with ubiquitin, an abundant cytoplasmic chaperone which target the molecule to proteasomes. The proteasomes degrade the proteins to amino acids or fragment them into peptides of varying length. The peptides generated by the proteasomes are translocated into ER by TAP proteins where they are delivered to the heavy chains of class I molecules (Monaco *et al.*, 1990; Neefjes *et al.*, 1993). Then, heavy chains bind to β2 microglobulin, the class I–peptide complexes are directed to the Golgi compartment, and finally to the cell surface where they are recognized by T-cells (Kleijmeer *et al.*, 1992). The peptides isolated from class I molecules are made of eight to ten amino acid residues (Rotzschke *et al.*, 1990). Both peptidic ends are inserted into the binding groove of the heavy chain of class I molecules.

A chimeric Ig expressing an epitope recognized by CD8 T cells should undergo an identical endogenous processing pathway to generate peptides sorted by class I molecules.

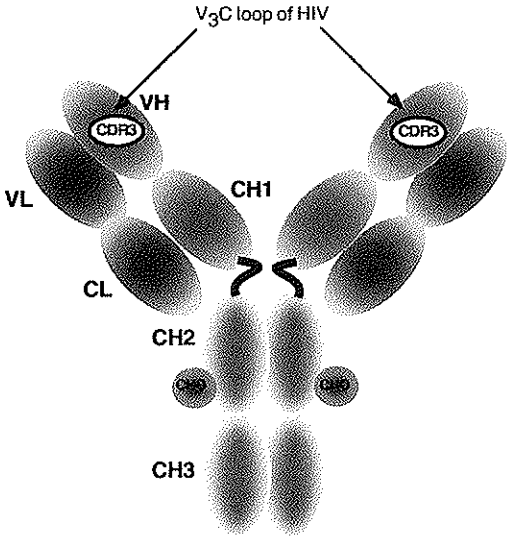

Figure 1. Schematic representation of Ig-V_3C chimeric molecule. The chimeric Ig-V_3C molecule was genetically engineered to express the V_3C peptide of the gp120 envelope protein of HIV-1 in the CDR3 loop of VH region of BALB/c IgG2b. The V_3C peptide (RKSIHIGPGRAFYTTGEII) corresponds to a consensus sequence predicted from the comparison of the V_3 cysteine bridged loop sequences of gp120 of 245 HIV-1 isolates. The BALB/c IgG2b represents the MOPC141 myeloma protein.

Zaghouani *et al.* (1992) prepared a murine chimeric Ig expressing a peptide corresponding to the 147–164 amino acid residues of the nucleoprotein (NP) of the PR8 influenza virus (IgNP). NP peptide is recognized by CTL in association with K^d class I molecules (Taylor *et al.*, 1987). IgNP was produced by the SP2/O myeloma cells cotransfected with a plasmid encoding for the VH(NP)-Cγ2b gene, and a plasmid encoding for the light chain gene.

The transfected cells secreting IgNP molecules were able to bind anti-NP, anti-Ig/k chain, and anti-γ2b antibodies. This demonstrated that transfected cells secreted a complete IgG2b molecule in which NP147–164 peptide was surface exposed.

Target cells preincubated with IgNP molecules were not killed by NP147–164 specific CTLs. In contradistinction, SP2/O transfected cells expressing IgNP genes were lysed. These observations suggested that while the processing of IgNP molecules occur by the endogenous pathway, thus generating NP147–164 peptide that is recognized by CTLs in association with K^d molecules, the internalized IgNP protein was not able to sensitize the target cells, presumably because was processed the exogenous pathway. Experiments carried out *in vivo* by Kuzu *et al.* (1993) supported this explanation.

The transfected cells expressing IgNP or VH(NP)-γ2b genes were able to induce *in vitro* a strong proliferative response of a NP147–161 specific CTL clone, in the presence of IL-2. In addition, when adult BALB/c mice were injected i.v. with irradiated transfected cells expressing the chimeric VH(NP)-γ2b gene, a NP147–161

CTL response was induced. It is noteworthy that SP2/O cells coated with NP147–161 peptide were unable to elicit a specific CTL response *in vivo* (*Table 2*). The ability of transfected cells expressing VH(NP)-γ2b and the inability of SP2/O cells coated with NP peptide to prime a CTL response may be related to the expression of higher amounts of class I-NP peptide complexes by transfected cells or to a higher stability of complexes consisting of newly generated class I heavy chains.

Table 2. Summarized results of the induction of proliferative response and of the *in vivo* priming of NP147–161 specific CTLs by IgNP

Antigen	*In vitro* proliferative response of NP147–161 specific T cells	*In vivo* priming of NP147–161 specific T cells
Nil	–[a]	–
PR8 virus	+	+++
NP coated SP2/0 cells	+	–
Transfectomas expressing:		
V_H-wild type gene	–	–
V_H-NP chimeric gene	+	++

[a] – to + stands for various magnitudes of response

Collectively, these data indicate that the translation product of a chimeric VH gene is processed via endogenous pathways and leads to the generation of a peptide that binds to class I antigens. This entire process mediates efficient recognition of the inserted peptide by the specific CTLs and exhibited the same antigenicity and immunogenicity as the NP peptide generated subsequent to antigen processing of the influenza virus nucleoprotein or the synthetic NP peptide itself. Because the NP synthetic peptide, and the NP peptide generated from the cells transfected with the chimeric VH(NP)-γ2b gene were efficiently presented by class I antigens to T-cells, it appears that the inability of presentation of NP peptide generated from the exogenously delivered IgNP molecules to the target cells or APCs, can be related to:

(i) the absence of trafficking of IgNP toward the ER may prevent the protein being processed through the endogenous pathway;
(ii) inability in recycling class I molecules (Tse-Eng and Pernis, 1984);
(iii) the lack of empty class I molecules in endosomes;
This is in agreement with data indicating a segregation of class I and class II MHC molecules in the post-Golgi apparatus. Whereas class I–peptide complexes found in vesicles are translocated to the surface via constitutive secretory pathway, the class II molecules are pumped towards lysosomes via *trans*-Golgi reticulum – like other endosomal proteins (Peters *et al.*, 1991).
(iv) the difference in fragmentation of the proteins by endosomal enzymes and proteosomes may lead to the generation of NP peptide recognized by CTLs within the cytoplasm, but not in lysosomes (Bona *et al.*, 1994).

SELF MOLECULES EXPRESSING FOREIGN PEPTIDES RECOGNIZED BY CD4 T CELLS

In contrast to CD8 cells that recognize the peptides generated via endogenous pathways in association with class I molecules, the CD4 T-cells recognize the peptides

generated in both exogenous and endogenous pathways in association with class II molecules (Babbitt *et al.*, 1985).

The exogenous pathway is a principal mechanism alerting the immune system to the presence of extracellular parazites, or soluble foreign molecules. The antigen degradation occurs in the acidic environment and is mediated mainly by acid proteases located in endosomes. The exogenous pathway mainly takes place inside the professional antigen presenting cells (pAPC) that constitutively express class II and costimulatory molecules (i.e. B-cells, macrophages and dendritic cells). Once the peptides are generated within the endosomes, they are sorted by class II molecules based on their affinity. Class II molecules are synthesized in ER. The appropriate conformation of the α and β chain heterodimer is dictated by the binding of the invariant chain (Ii, also called the γ chain). While the class I molecules are pulled out to the cell surface through the trans-Golgi region, the Ii, a chaperone molecule, targets the class II molecules to the endosomal compartment via Golgi apparatus. In endosomes, the Ii chain is degraded and the class II molecules bind the peptides generated from the processing of foreign molecules. The new complex then is translocated to the surface of the APC. The efficiency of presentation of a peptide recognized by CD4 T cells depends on several factors such as the access of the antigen in specialized endosomal vesicles, the arsenal of endosomal enzymes, the availability of class II molecules that can sort a given peptide and the affinity of the newly generated peptide for the empty class II molecules. A correct understanding of the molecular mechanisms underlying the exogenous pathway is required for the evaluation of the immunogenicity of chimeric self molecules expressing foreign peptides.

Zaghouani *et al.* (1993b) designed a chimeric Ig bearing a virus class II restricted epitope by replacing the CDR3 segment of an anti-arsonate IgG2b antibody with an influenza virus sequence encoding HA110–120 peptide. This peptide is a dominant T-helper epitope recognized in the context of murine I-E^d histocompatibility molecules (Haberman *et al.*, 1990). The chimeric Ig named IgHA (*Figure 2*), activated TcH specific for HA110–120 in context of I-E^d. Two distinct TcH were used to validate the activity of IgHA: (i) LD1-24 TcH that produces IL-3 following the TCR engagement (Haberman *et al.*, 1990) and (ii) 14-3-1 TcH that expresses β-galactosidase under the IL-2 promoter following the activation (Bot *et al.*, 1996). As shown in *Table 3*, three types of APC – namely irradiated splenocytes, splenic dendritic cells and 2PK3 B lymphoma cells, were used to estimate the ability of IgHA to activate TcH compared to the synthetic HA110–120 peptide and the bromelain-released haemagglutinin (BHA). The potency of IgHA was 100–1000 times higher relative to the synethetic peptide, by comparing the molar amount of antigen required to induce 50% of the maximal response. The BHA was equal or slightly more potent than IgHA on molar basis. Significantly, this hierarchy was not dependent on the type of TcH used as read-out or the type of APC pulsed with antigen (Zaghouani *et al.*, 1993b; Bot *et al.*, 1996).

Further experiments were aimed at defining the mechanisms by which IgHA generates the immunogenic peptide in context of class II molecules. Pretreatment of APC with a high affinity mAb specific for FcγR greatly reduced the ability of the IgHA pulsed APC to activate specific TcH (Zaghouani *et al.*, 1993b). In contrast, control IgG antibodies displayed reduced inhibitory ability and IgM antibodies or Fab fragments of IgG antibodies showed no inhibition. Significantly, even large concen-

trations (up to 100 μg/ml) of high-affinity FcγR specific mAb (i.e. 2G4-2) could not totally prevent a degree of TcH activation. These data suggested that IgHA is taken up in the APC through two pathways: (i) a fast, efficient pathway dependent on the engagement of FcγR and (ii) a slow, less effective mechanism probably mediated by fluid-phase pinocytosis.

Table 3. The relative immunopotency of IgHA assessed by *in vitro* stimulation of TcH specific for HA110–120 peptide in the context of I-E^d class II molecules expressed by various antigen presenting cells (APC)

Antigen presenting cells	NP147–155	IgG2b	HA110–120	IgHA	BHA
Splenocytes[b]	–[a]	–	+	+++	++++
Dendritic cells[b]	–	–	+	+++	++++
B lymphoma cells[c]	–	–	+	+++	++++

[a]Comparison based on the molarity required for 50% activation.
[b]APC were incubated with LD1-24 TcH in the presence of various concentrations of antigens. IL-3 production was estimated as described by Zaghouani *et al.* (1993).
[c]2PK3 B lymphoma cells were incubated with 14-3-1 TcH in the presence of various concentrations of antigens. The percentage of activated TcH was estimated as described by Bot *et al.* (1996).

Pretreatment of APC with chloroquine or paraformaldehyde fixation abolished their ability to process IgHA and to present the HA110–120 peptide. As expected, the pretreatment of APC with chloroquine or paraformaldehyde did not prevent the presentation of the synthetic HA110–120 peptide to specific TcH.

Two lines of evidence suggested that HA110–120 peptide generated from the processing of IgHA was presented in the context of class II I-E^d molecules: (i) anti-I-E^d but not anti-I-A^d antibodies inhibited the activation of TcH by IgHA pulsed APC (Zaghouani *et al.*, 1993b) and (ii) HA110–120 was eluted from class II molecules of IgHA pulsed B lymphoma cells (Brumeanu *et al.*, 1993). Together, these results showed that IgHA was internalized through FcγR into the endosomal compartment where it is degraded by acid proteases and the resultant HA110–120 peptide is bound to class II molecules. The class II–peptide complex is translocated to the surface of APC where it is recognized by the specific TCR. Thus, the processing pathway resembles that of virus or BHA, with the important difference that the receptors which mediate the uptake are different, i.e. the sialoreceptors in the case of BHA and virus. In contrast, the HA110–120 synthetic peptide directly binds to the few empty class II molecules at the surface of APC. Because the loading of the class II molecules occurs mostly in the CII vesicles where Ii is shed off from the class II heterodimer, the result is that effective strategies of targeting helper epitopes to class II molecules ought to lead to the delivery of the foreign peptides into the endosomal compartment. This finding can explain the increased immunopotency of IgHA, BHA or the virus itself, compared with the HA110–120 synthetic peptide.

Experiments aimed at estimating the persistence of the immunogenic class II–peptide complexes on the surface of APC following the epitope delivery through IgHA, BHA, PR8 virus or synthetic peptide showed that the half-life was identical to the half-life of MHC-II molecules, approximately 50 hours (Bot *et al.*, 1996). This result is concordant with previous estimations of the half-life of class II–peptide complexes (Lanzavecchia *et al.*, 1992) and strongly suggests an irreversible binding

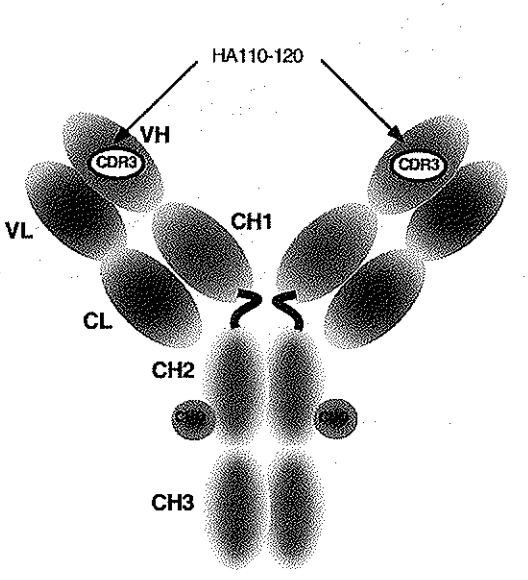

Figure 2. Schematic representation of Ig-HA chimeric molecule. The chimeric IgHA molecule was genetically engineered to express the HA110–120 peptide of PR8 virus in the CDR3 loop of V_H region of BALB/c IgG2b. The HA110–120 (SFERFEIFPKE) is the CD4 T cell immunodominant epitope of the haemagglutinin of the A/PR/8/34 influenza virus. The BALB/c IgG2b represents the MOPC141 myeloma protein.

of immunogenic peptides to class II molecules. Thus, whereas the processing time depends on the carrier, the persistance of class II–peptide complexes is a constant depending on the half-life of class II molecules (Bot *et al.*, 1996) (*Table 4*).

Table 4. Kinetics of generation and persistence of the immunogenic HA110–120 peptide on class II molecules of a B cell lymphoma APCs

Antigen	Generation of class-II peptide complexes (time required for 50% of maximal activation)[a]	Persistence of class-II peptide complexes (time required for 50% of maximal activation)[b]
HA110–120	< 15 minutes	50 hours
IgHA	2 hours	50 hours
BHA	1 hour	50 hours
PR8 virus	1 hour	50 hours

[a]APCS were pulsed with antigens for various intervals of time, washed extensively, and reincubated with TcH for optimal periods of time.
[b]APCs were pulsed with antigens for optimal intervals, washed extensively, and incubated for various intervals of time. Persistence of the immunogenic HA110–120/class II complexes was assessed following the addition of specific TcH, by measuring the T-cell activation.

Antibodies to the carrier can modulate the immunogenicity of class II restricted epitopes in various and often unpredictable ways (Simitseck *et al.*, 1995). It was found that anti-HA110–120 antibodies greatly decreased the immunogenicity of IgHA. This

was due to a decreased generation of the class II–peptide complex inside the endosomal compartment rather than preventing the uptake or the engagement of class II molecules by TCR (Bot *et al.*, 1996). Thus, factors apparently independent of the internalization can dramatically affect the immunogenicity of a given epitope borne by a carrier.

IgHA chimera induced a significant immune response when administered subcutaneously to BALB/c mice, in CFA. Doses of 150μg of IgHA primed immune responses of higher magnitude than equivalent doses of HA110–120 synthetic peptide, containing approximately 100 times more epitope on a molar basis (Zaghouani *et al.*, 1993b). Furthermore, subcutaneous inoculation of TCR-HA transgenic mice expressing a TCR specific for HA110–120 on I-E^d molecules (Kirberg *et al.*, 1994), with 100 μg IgHA in CFA followed by a saline boost, led to the expansion of the CTL precursors pool specific for the HA110–120 peptide (*Table 5*). In contrast, the synthetic peptide was less effective in increasing the HA110–120 specific pCTL frequency. The PR8 virus and a reassortant bearing the HA110–120 epitope (P50 virus) did not significantly increase the peptide-specific pCTL frequency. This can be explained by the simultaneous expansion of T-cells specific for other antigens borne by the viruses and is supported by the fact that HA110–120 specific pCTL frequency is significantly reduced following the immunization with BLee that does not carry the HA110–120 peptide (Brumeanu *et al.*, 1997).

Table 5. Immunogenicity of IgHA in TCR-HA transgenic mice

Antigen[a]	Frequency[b]	Frequency^{-1}	Relative frequency[c]
IgG2b	3.8×10^{-4}	2630	1.0
IgHA	2.8×10^{-3}	360	7.3
HA110–120	4.3×10^{-4}	2330	1.1
P50 virus	4.2×10^{-4}	2380	1.1
BLee virus	1.3×10^{-4}	7690	0.3

[a]TCR-HA Tg mice were immunized subcutaneously with 100μg of antigen in CFA and boosted intraperitoneally, 2 weeks later with 100 μg of antigen in saline. Mice were sacrificed 1 week after the boost.
[b]The frequency of HA110–120 specific pCTLs in the spleen was estimated as previously described by Brumeanu *et al.* (1997).
[c]The relative frequency was expressed using as reference mice injected with IgG2b.

In conclusion, subcutaneous inoculation of IgHA in moderate doses is followed by significant expansion of HA110–120 specific T-cells in BALB/c as well as in the TCR-HA transgenic mice. Even higher quantities of HA110–120 synthetic peptide, on a molar basis, were not as effective as the IgHA in that respect. This strongly supports the results of *in vitro* studies and suggests that the increased immunogenicity of IgHA is determined by the preferential targeting of the chimera to the endosomal compartment of bone marrow derived APC, that express Fcγ receptors. In contrast, most of the synthetic peptide is rapidly degraded by serum proteases or internalized by fluid-phase pinocytosis in non-professional APC.

Lanoue *et al.* (1997) studied the conditions that induce tolerance of the mature CD4 T-cells from TCR-HA transgenic mice. Repeated inoculation of IgHA in saline (intravenous injection, five times every other day at 100 μg/dose) was followed by a significant decrease in the number of 6.5^+ T-cells bearing TCR transgene, both in the

thymus and peripheral lymphoid organs. In contrast, the HA110–120 synthetic peptide was less effective in inducing peripheral anergy and depletion of TCR^+ T-cells. The relative lack of effectiveness of the synthetic peptide in inducing tolerance following repeated inoculation is probably due to the rapid clearance of the peptide, resulting in a decreased loading of the class II molecules on APC. Two possible mechanisms that do not exclude each other may account for the increased effectiveness of IgHA in inducing peripheral tolerance upon repeated inoculation: (i) an effective targeting of the epitope to class II molecules on professional APC followed by overstimulation of T-cells and apoptosis and (ii) targeting of the epitope to a broader category of APC which, in spite of expressing FcγR and class II molecules, lack optimal expression of costimulatory molecules (i.e. small, resting B-cells). The enhanced ability of IgHA to prime or in distinct circumstances, to tolerize specific T-cells, may stem from the same property, namely to effectively address the endosomal loading pathway of class II molecules.

Legge *et al.* (1997) used the same strategy to construct Ig chimeras expressing non-Ig self epitopes or modified self epitopes that can modulate the activity of specific T cells in an antagonistic manner. The CDR3 of the heavy chain of the same anti-arsonate antibody was replaced with an antagonistic variant of the PLP1 peptide obtained by mutating the TCR contacting residues (Ig-PLP-LR) (Kuchroo *et al.*, 1994). The main hypothesis was that, in order to effectively antagonize pathogenic epitopes derived from autoantigens (namely PLP1 derived from the PLP protein) within the endosomal compartment of APC, the antagonistic peptides (i.e. PLP-LR) should be delivered by a carrier in the same compartment. Ig-PLP-LR effectively inhibited the IL-2 production of TcH stimulated with APC pulsed with the PLP1 peptide, PLP protein or Ig-PLP. Whereas the antagonistic peptide PLP-LR, inhibited to a certain extent the activation of TcH triggered by PLP peptide, it failed to inhibit the activation of TcH by Ig-PLP or the PLP protein. Furthermore, *in vivo* experiments showed that, whereas Ig-PLP-LR greatly prevented the generation of PLP1 specific T-cells following the immunization with Ig-PLP1, the synthetic antagonistic peptide was significantly less potent.

In view of some recent studies, two mechanisms that are not mutually exclusive may account for the antagonistic activity of the Ig-PLP-LR chimera (i) engagement of the PLP1 specific T cells by class II-PLP-LR complexes on APC leading to the generation of a negative signal (Lyons *et al.*, 1996) and (ii) induction of a suppressor subset specific for the PLP-LR but not PLP1 peptide (Nicholson *et al.*, 1996). The enhanced *in vitro* and *in vivo* antagonistic effectiveness of the Ig-PLP-LR chimera compared to the antagonistic peptide (PLP-LR) is most probably due to efficient targeting of the molecule to the endosomal compartment of APC followed by an increased loading of empty class II molecules.

ENZYMATICALLY ENGINEERED *SELF* IMMUNOGLOBULINS EXPRESSING FOREIGN PEPTIDES

The most attractive feature of the enzymatic reactions is their high specificity for the substrate. Enzymes require restricted sites of recognition on the substrate, such as peptide bonds, particular amino acid residues or even a specific chemical group. In addition, to be active, many enzymes require an extremely well-defined conforma-

tional structure which is stable in a limited range of pH. This is the case for galactose oxidase (GAO), a liver enzyme able to oxidize at pH 7–8 the hydroxyl group of the fourth carbon of the galactose ring. The oxidation takes place only when the galactose is located in a terminal position (Avigad, 1985). The very confined specificity GAO is also related to the conformational integrity of the carbon 5, 7 and 8 on the galactose ring (Avigad, 1985). This allows GAO's catalytic site to differentiate between galactose and glucose. The remarkable specificity of GAO, made this enzyme useful in clinical tests to distinguish between the blood levels of galactose vs glucose, in patients with pathological sugar-related conditions. Early approaches using GAO reaction, showed the possibility of labeling soluble and cell surface glycoproteins with radioisotopes (Morell *et al.*, 1972; Mitchell *et al.*, 1984).

Like many self proteins, immunoglobulins (Ig) undergo postranslation modification to attach to sugar units of N-glycan-like structure in which the galactose residues are located subterminal to the sialic acid (Harada *et al.*, 1987). On immunoglobulins, these sugar units are mainly bound through the asparagine residue to CH2 domain of the Fc fragment (Harada *et al.*, 1987). Generally, the IgG molecules express a single N-glycan unit on each CH2 domain, and each N-glycan contains two galactose residues located adjacent to the terminal sialic acid (Corfield*et al.*, 1983; Weitzhandler *et al.*, 1994). The IgA and IgM molecules may express between 3 and 12 N-glycan chains located in various positions on CH1, CH2 and CH3 domains (Endo *et al.*, 1994).

Glycosidic assembly of the viral peptides on self *immunoglobulins*

Taking advantage of the fine specificity of GAO to oxidize the galactose residues of N-glycans on the Igs, and also aiming at the generation of a novel delivery system for foreign peptides by *self* Igs, Brumeanu *et al.* (1995) developed the methodology of enzymatic mediated assembly of synthetic peptides on the sugar moieties of various mouse and human Ig isotypes. As illustrated in *Figure 3*, the synthesis of immunoglobulin-galacto-peptide constructs (IGP) consists in galactose oxidation by GAO of the desialylated Igs followed by covalent attachment of the peptides with concurrent stabilization of the imidic bonds upon mild reduction with pyridine borane.

This methodology was found efficient in peptidizing Igs (*Figure 4*) because of several advantages:

(i) the branched architecture of the sugar moiety contains usually four galactose acceptors per molecule of IgG and up to 20 per molecule of IgM. Estimation of the efficacy of coupling of an influenza virus haemagglutinin peptide showed an average of 11.4 peptide/IgG1 and 3.4 peptides/IgG2b molecules;

(ii) the high specificity of coupling reaction is conferred by GAO reaction. Thus, the deglycosylation of IGPs with PGN-ase was able to cleave specifically the N-glycans (*Figure 5*) indicated that GAO mediated the coupling of peptides exclusively to the carbohydrate moieties of Igs;

(iii) the enzymatic coupling did not alter the biological properties of the peptide attached to Ig, nor those of the Ig itself;

(iv) the lack of chemical cross-linkers between the peptide and Ig avoids the

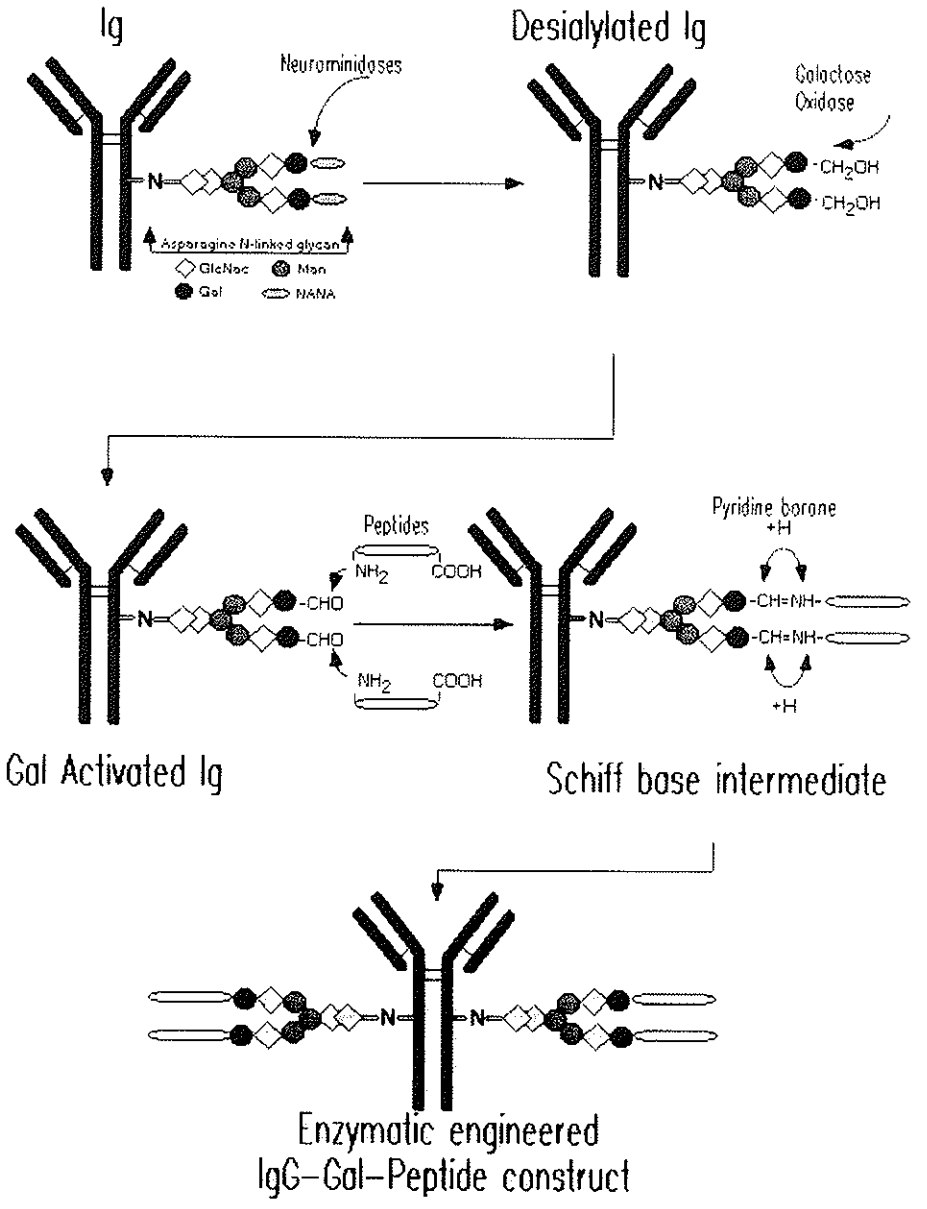

Figure 3. Enzymatic synthesis of immuno-galacto-peptide constructs (IGP). The sialic acid residues in the terminal position of the N-glycans of Igs are removed by treatment with neuraminidases from *Clostridium* and *Arthrobacter*. The adjacent galactose residues are then oxidized with galactose oxidase, and the α primary amino group at the N-terminus of the synthetic peptides generate instantaneously an intermediate Schiff base that is concomitantly stabilized with pyridine borane.

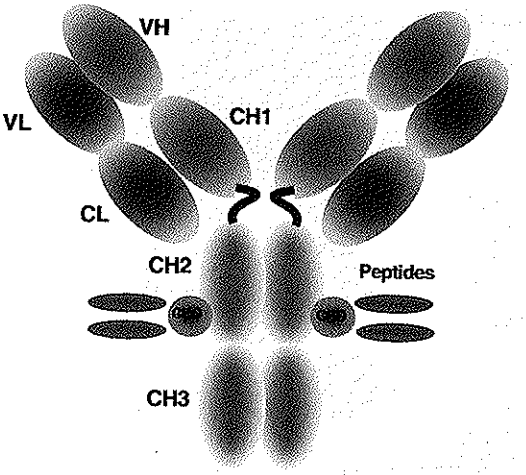

Figure 4. Schematic representation of Ig-gal-HA construct. The IgG-gal-HA110–120 construct was enzymatically engineered to express the immunogenic peptides on the galactose residues of the N-glycans of IgG, according to the synthesis process described in Figure 3.

induction of neodeterminants following immunization, as it is well known in the case of chemically synthesized protein–peptide conjugates;

(v) the method is simple, rapid, and the IGP constructs can be easily purified from the reaction mixture by standard chromatographic techniques.

Using this method, several IGPs carrying either a CD4 T cell immunodominant epitope HA110–120 or the major B cell epitope HA150–159 of the haemagglutinin (HA) of the A/PR/8/34 influenza were generated in our laboratory (Brumeanu *et al.*, 1995; Brumeanu *et al.*, 1996a).

Activation of the cognate T-cell subsets by IGP constructs

To test the ability of IGP constructs in activating *in vitro* the peptide-specific T cells, several mouse IgG and IgM constructs carrying the HA110–120 CD4 T-cell immunodominant epitope of HA of the A/PR/8/34 influenza virus were investigated. A mouse IgG-gal-HA and IgM-gal-HA was able to activate the HA110–120-specific T-cell hydriboma LD1-24 at levels comparable to the genetically engineered Ig-HA chimeric molecules and UV-irradiated PR8 influenza virus, when the readout system was the MTT colorimetric assay (Mosmann, 1983). This was the first indication that foreign peptides assembled on the sugar moieties of Igs are immunogenic.

To investigate the ability of IGP constructs in priming precursors of the cognate T-cells, Brumeanu *et al.* (1996b) determined the proliferative response of lymph nodes from BALB/c mice immunized with a mouse IgG-gal-HA110–120 or IgM-gal-HA construct. Data summarized in *Table 6* show that priming of mice with these constructs stimulated the proliferation of cognate T-cells following challenge *in vitro*

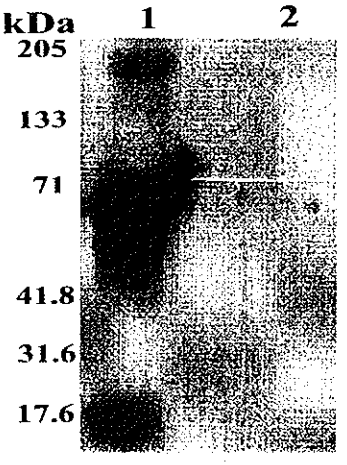

Figure 5. Specificity of galactose-peptide linkage. IGP constructs were either non-treated (lane 1), or treated (lane 2) with PGN-ase, an enzyme able to cleave the entire N-glycan of Igs at the asparagine-N bond. Samples were then electrophoresed in 4–15% gradient of polyacrylamide under denaturing and reducing conditions, gels were electrotransferred on PVDF membranes, and the N-glycan bound HA110–120 peptides were revealed with a rabbit anti-HA110–120 Abs (Ab1) followed by ^{125}I-labelled goat anti-rabbit IgG Abs (Ab2). PGN-ase was able to remove entirely the N-glycan-peptide complexes from an IgG2b-gal-HA110–120 construct, indicating the strict specificity of the coupling reaction during the enzymatic synthesis of IGP.

with HA110–120 peptide. This was an indication that IGP constructs carrying foreign peptides on the sugar moieties of Igs, are able to stimulate *in vivo* the cognate T-cell precursors.

Table 6. *In vivo* priming of peptide-specific T cell precursors by IGP constructs

In vivo priming with:	*In vitro* challenge with:						
	ConA (2μg/ml)	PPD (0.1μg/ml)	HA110–120 (80nm)	HA150–159 (80nM)	PR8 virus (10μg/ml)	BLee virus (10μg/ml)	BHA (1.1μg/ml)
	^{3}[H]-TdR incorporation (cpm)[a]						
IgG2b (control)	24 500	12 300	457	378	623	512	650
Ig-HA	12 800	7 850	10 980	1 050	11 967	1 023	12 560
IgG2b-gal-HA110–120	14 800	6 890	13 100	980	9 870	860	15 200
IgM (control)	13 890	6 650	629	570	735	850	724
IgM-gal-HA110–120	8 500	6 050	14 070	815	11 820	760	11 890
HA110–120	11 050	3 815	1 012	320	1 820	815	540

[a]The proliferative response of lymph nodes from BALB/c mice immunized with various genetically and enzymatically constructs, PR8 virus and Blee virus control, was assessed by thymidine incorporation assay. Counts (cpm) represent the mean of triplicate wells.

To find out whether the IGP constructs are also able to activate resting T-cells, Brumeanu *et al.* (1996a) used as a readout system, transgenic mice in which the TCR is specific for HA110–120 peptide in association with I-E^d class II molecules is expressed on both CD4 and CD8 T cell subsets (Kirberg *et al.*, 1994). The

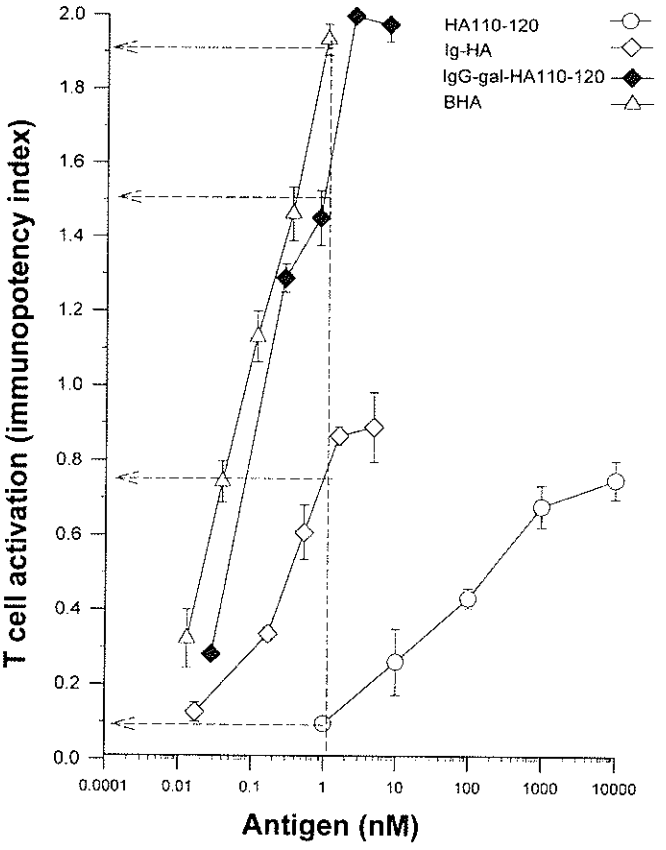

Figure 6. Immunopotency of HA110–120 epitope delivered by various carriers. Irradiated 2PK3 APCs were incubated with LD1-24 TcH specific for HA110–120 peptide for 48 h with various amounts of the following antigens: HA110–120 synthetic peptide (SFERFEIFPKE), IgHA genetically engineered chimeric molecules. IgG2b-gal-HA110–120 enzymatically engineered construct, and bromelain released protein from the haemagglutinin (HA) of the A/PR/8/34 influenza virus. The culture supernatants were assessed by the MTT assay for the production of IL-3, using the DA-1 IL-3 dependent cell line. The activation values were expressed in OD units at 570 nm. The specificity controls were HA150–159 (WLTEKEGSYP) B cell epitope of HA of the PR8 influenza virus and the murine IgG2b MOPC141. The immunopotency of various carriers to deliver HA110–120 epitope was evaluated by comparing the T cell activation indexes (Y axis) by 1 nM (calculated) of peptide delivered by various carriers. T cell activation indexes were calculated according to the ratio between the activation value (OD570 nm) subsequent stimulation with HA110–120 carriers vs the activation values obtained with the specific controls. Dotted lines represent the level of activation obtained with 1 nM of HA110–120 peptide as delivered by various carriers. Samples were measured in quadruplicate wells and the +/– SD was calculated.

cytofluorometric analysis using double staining of the transgenic T-cells with both 6.5.2 anti-TCR-HA clonotypic antibody and anti-CD25 (IL-2R) mAb AMT-13 showed that these cells are not activated (Brumeanu *et al.*, 1996a). *In vitro* incubation of negatively selected $CD4^+/CD8^-/CD25^-$ and $CD4^-/CD8^+/CD25^-$ HA110–120-specific T-cells, with irradiated spleen cells from BALB/c mice as APCs, showed a strong proliferative response upon exposure to IgG-gal-HA110–120 construct. IgG-gal-HA110–120 was able to induce three-fold higher proliferative response of the resting T-cells than the genetically engineered Ig-HA, UV-inactivated PR8 virus and BHA.

Furthermore, the immunopotency of HA110–120 peptide expressed on various carriers was evaluated by measuring their capacity to activate cognate T-cells. For this, the amount of peptide per carrier was normalized based on molar equivalents. Thus, 1 nM of HA110–120 peptide is carried by 0.35 nM IgG-gal-HA, 0.5 nM chimeric Ig-HA and 0.3 nM bromelain released haemagglutinin (BHA). As illustrated in *Figure 6*, the peptide linked to the sugar moieties of a mouse IgG2b was as potent as BHA, two times more potent than Ig-HA, and about 20 times more potent than HA110–120 synthetic peptide.

When the frequency of T-cell precursors induced by IgG-gal-HA110–120 construct was compared with that induced by P50 influenza virus, a reassortant sharing only the HA gene with PR8 virus, the construct showed significant higher values than the virus (*Table 7*). This may be explained by the fact that the immune response elicited by antigenized self Igs is restricted to the foreign epitopes, unlike that for the activated virus which bears a myriad of irrelevant epitopes.

Table 7. HA110–120-specific T cell frequency[a] induced in TCR-HA transgenic mice by IgG-gal-HA110–120 construct

Antigen	Frequency (∂)	$1/\partial$
mouse IgG2b	3.8×10^{-4}	2 650
mouse IgG2b-gal-HA110–120	1.1×10^{-2}	90
P50 virus	4.2×10^{-4}	2 400
BLee virus (control)[b]	1.2×10^{-4}	8 330

[a]The frequency of T cell precursors was calculated as described by Brumeanu *et al.* (1997);
[b]BLee virus (control) does not express the HA envelope protein of the A/PR/8/34 influenza virus.

Mechanism of antigen presentation of peptides linked to the sugar moieties of self *immunoglobulins*

To investigate why the immunopotency of HA110–120 peptide linked to the sugar moieties of self Igs is higher than that of other carriers, there was analysis to see whether the sugar-linker moiety can release the peptide in the lysosomal compartment of the antigen processing cells (Brumeanu *et al.*, 1995). For this, two variants of IGP constructs were generated: IgG-gal-HA110–120 in which the peptide was attached enzymatically to the galactose residues of a mouse IgG2b as 11-mer, and IgG-gal-HAc-110–120 in which the amino terminal of HA110–120 peptide coupled to the galactose residues contained the cathepsin D catalytic site made of Ala-Ala-Ala-Leu tetrapeptide. It is known that cathepsin D represents the major proteolytic lysosomal enzyme responsible for the antigen processing of foreign proteins (Yonezawa *et al.*, 1987). No significant difference in the capacity of activating LD1-24 TcH was observed between the two constructs. This was an indication that the activation of T-cells is not dependent on the attachment of peptides on the sugar moiety of a particular Ig isotype. In the case of HA110–120 linked to the sugar moieties of IgM, it was found that processing of the IgM-gal-HA110–120 construct lead to generation of HA peptides with structures similar to the canonical synthetic peptide, as well as that generated from processing of Ig-HA or PR8 virus (*Table 8*).

Table 8. Structure of the HA peptides extracted from I-E^d molecules of 2PK3 APCs pulsed with chimeric self molecules carrying the HA110–120 peptide

10^9 2PK3 cells pulsed with 30 mg of:	Amino acid sequence of HA peptides recovered from I-E^d molecules	Yield (pM)
HA110–120	SFERFEIFPKE	102
IgM-gal-HA110–120	SFERFEIFP	325
	SFERFEIF	123
Ig-HA	SFERFEIFPKE	470
	SFERFEIF	30
PR8 virus	SFERFEIFPKE	10

The yield of the peptides extracted from I-E^d molecules was calculated using the repetitive yield analysis in a protein gas sequencer (Porton Instruments, CA).

In aggregate, these results support several conclusions: (i) the sugar moiety of Igs offers a good platform for delivery of foreign peptides; (ii) depending upon the conformation of the sugar, the linked peptide may be, or may be not the subject of proteolytic degradation in APCs, and (iii) the yield of loading class II molecules with a minimal length epitope may play an important role on the immunopotency of IGP constructs.

In contradistinction, it was found that the IgG-gal-HA110–120 construct was able to activate the cognate T-cells without intracellular or extracellular processing. The presentation may also occur following the binding of the construct to FcγR on the surface of APCs, with concurrent interaction of the peptides to their neighbouring MHC class II molecules (*Figure 7*) (Brumeanu *et al.*, 1997). Cell-free processing mechanism of peptide presentation may also explain the high immunopotency of the IgG-gal-HA110–120 construct. Thus, the IGP constructs may harness the immune response *in vivo* by the engagement of APCs with low capacity of antigen processing, such as neonatal B-cells.

PIONEERING STUDIES ON THE PEPTIDIZED *SELF* GLYCOSAMINOGLYCANS WITH VIRAL EPITOPES

Glycosaminoglycans (GAG, proteoglycans) are large complexes of negatively charged carbohydrate chains generally associated with a small amount of protein core (Champe and Harvey, 1987). These compounds have the special ability to bind large amounts of water, thereby producing the gel-like matrix that forms the basis of the body's ground substance. Connective tissues, such as the skin, tendons, cartilage, ligaments, the matrix of bone, and teeth, comprise insoluble protein fibres distributed in the extracellular matrix.

Among these compounds, there are six major GAG classes, categorized according to their monomer compositions, type of glycosidic linkage, and degree and site of their sulphate groups. The sugar content of Chondroitin 4- and 6-sulphate contains disaccharide units made of N-acetylgalactosamine and glucuronide, with the sulphate group on C-4 or C-6 of the acid. Chondroitin sulphates are the most abundant GAG in the body, and are found in cartilage, bone and heart valves. Dermatan sulphate contains disaccharide units made of N-acetylgalactosamine and L-iduronic acid with variable amounts of glucuronic acid. It is found in skin, endothelial cells and heart

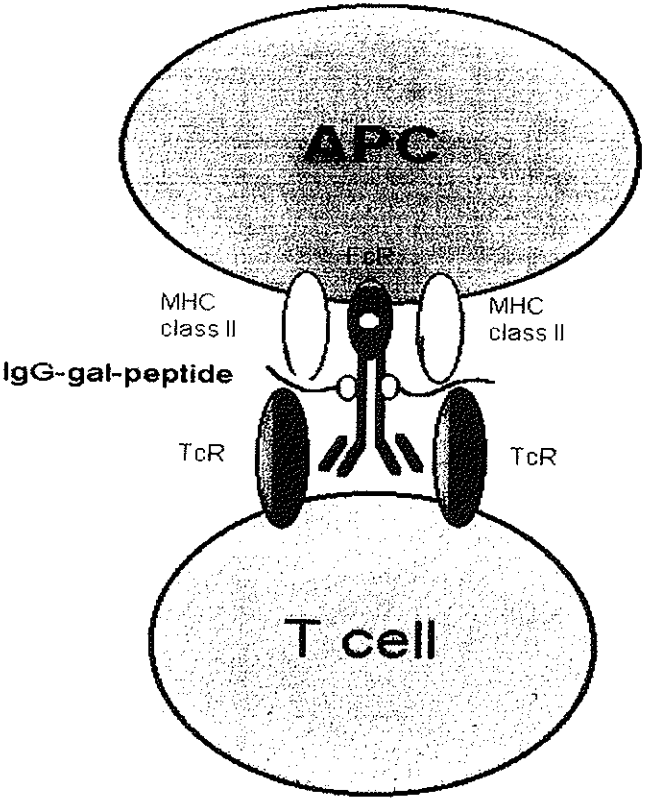

Figure 7. Cell-free processing mechanism of peptide presentation by IgG-gal-peptide constructs to T-cells. The presentation occurs following the binding of IgG-gal-HA construct to FcγR on the surface of APCs, with concurrent interaction of the peptides to their neighboring MHC class II molecules. Presumably, the conformational structure of the IGP allows two peptides to associate with two class II molecules which then may engage two TCR molecules in close proximity on the surface of a single T-cell.

valves. Keratan sulphate contains disaccharide units made of N-acetylglucosamine and a variable amount of sulphate present on C-6 of each of the sugar units. It is found in cartilage proteoglycan aggregates, together with Chondroitin sulphate.

GAG-peptide constructs were also prepared using the conventional perioxidation method. GAGs, such as chondroitin-sulphate, dermatan-sulphate, keratan-sulphate, or glycogen, were coupled to HA110–120 peptide of the A/PR/8/34 influenza virus. These constructs activated the cognate TcH 14-3-1 which express the 14.3 d specific TCR for HA110–120 peptide in association with I-E^d class II molecules (*Figure 8*).

GAG compounds can offer several advantages of immunological interest:

(i) GAG as *self* glycoproteins, are not expected to elicit anti-carrier antibody;
(ii) the high sugar content of GAGs provides a large number of acceptor sites for foreign peptides;
(iii) the synthesis of GAG–peptide constructs is simple and rapid, and the purification process requires only dialysis;

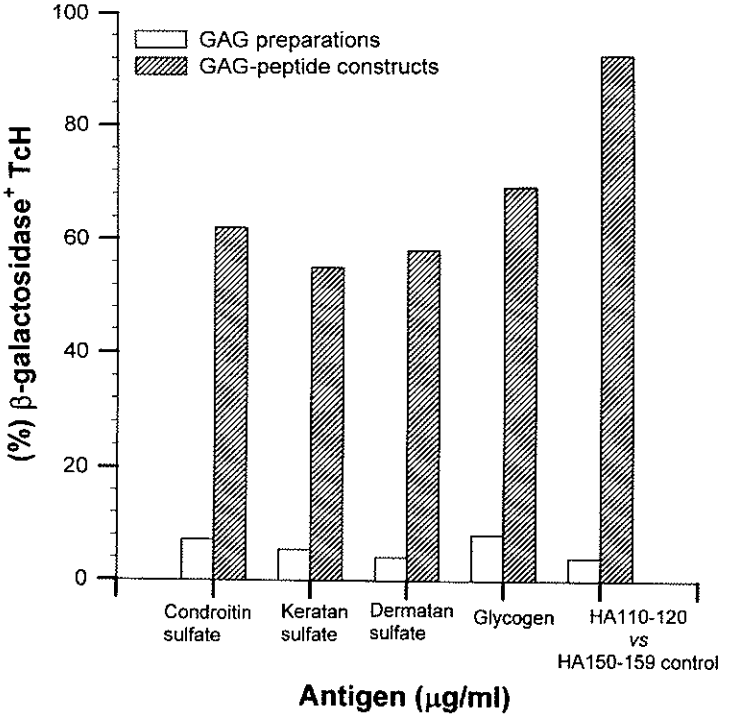

Figure 8. T-cell activation by Glycosaminoglycan-peptides and Glycogen-peptide constructs. Condroitin sulphate, keratan sulphate, dermatan sulphate and glycogen were glycosidically coupled with the HA110–120 T-cell immunodominant epitope of HA of the PR8 influenza virus, and the purified constructs (10 μg/ml) were incubated with BALB/c APCs and the 14-3-1 TcH specific for HA110–120 peptide in association with I-E^d class II molecules. After 24 h, the percentage of β-galactosidase positive TcH was estimated by FACS analysis among 500 events.

(iv) the remarkable long life of GAG in serum (i.e. keratan sulphate, 120 days) may prolong the time of priming *in vivo* the immunocompetent cells.

Boons *et al.* (1991) showed that a synthetic sugar–peptide conjugate was able to elicit T-helper response against *Neisseria meningidis*. Preliminary results using GAG-HA110–120 constructs indicate that the foreign peptides linked to the sugar moieties of GAG, vigorously stimulated the HA110–120-specific T-cells.

DOUBLY ANTIGENIZED Ig MOLECULES BEARING T AND B CELL EPITOPES

Immune responses elicited by T-dependent antigens require the co-operation between T- and B-cells (Mitchison, 1971). Generally, the epitopes recognized by T- and B-cells are structurally different in spite of the fact that they are borne by the same antigen unit.

A novel approach referred to as a 'multipeptide system' which consists of building multiple epitopes on branched lysine residues during the peptide synthesis, was carried out (Tam and Lu, 1989). Another approach was the synthesis of contiguous T-B epitopes linked, or not, by short peptide linkeres. Brumeanu *et al.* (1997) showed

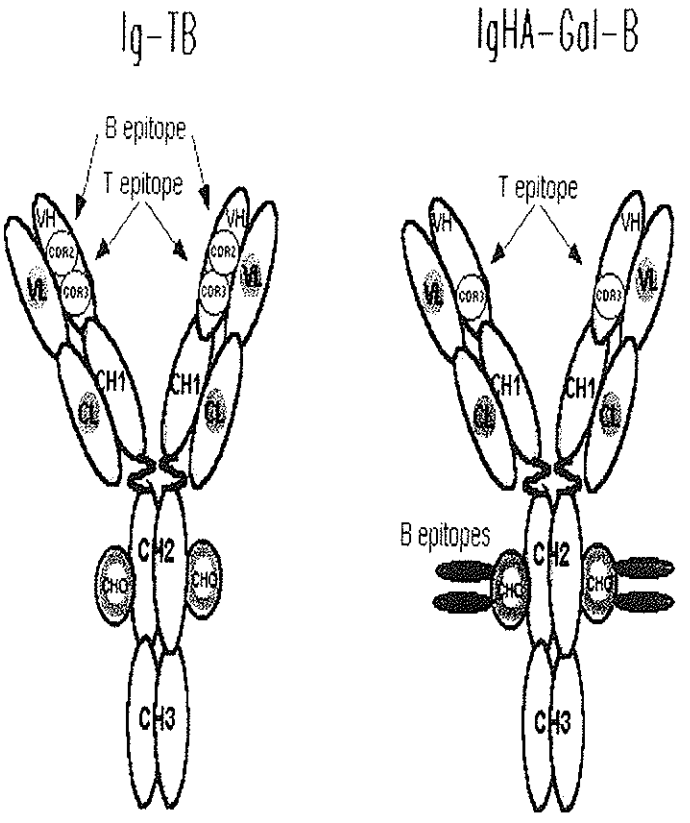

Figure 9. Schematic representation of doubly antigenized self Igs generated either by genetic engineering, or by a combination of the genetic and enzymatic engineering approaches. *Left panel* represents the genetically engineered Ig-TB chimeric molecule expressing the HA110–120 T cell epitope of HA of the PR8 virus in the CDR3 region of V_H of Ig, and the HA150–159 B cell epitope of HA of the PR8 virus in the CDR2 region of V_H of Ig. *Right panel* represents the combined genetically/enzymatically engineered Ig-HA-gal-B construct expressing the HA110–120 T cell epitope of HA of the PR8 virus in the CDR3 region of V_H of Ig, and the HA150–159 B cell epitope of HA of the PR8 virus linked to the sugar moieties of Ig.

that in a contiguously linked T-B synthetic peptide composed of two immunodominant epitopes of influenza virus, one was recognized by CD4 T-cells (HA110–120) and the other one which was recognized by B-cells (HA150–159), was able to induce strong antiviral antibody titers and a high frequency of specific T-cells.

These two immunodominant peptides were also expressed into Ig molecules. Two doubly antigenized Ig molecules were prepared (*Figure 9*).

One chimeric molecule designated IgT,B, was obtained by replacing the CDR2 loop of the VH gene with the HA150–159 B epitope and the CDR3 with HA110–120 CD4 T-cell epitope.

The second chimeric molecule designated IgHA-gal-B, consists of genetically engineered IgHA molecule in which the B cell epitope was coupled to the sugar moiety (Brumeanu *et al.*, 1996b).

The data presented in *Table 9* summarize the results obtained from Western blotting

analysis and RIA, demonstrating that both B- and T-cell epitopes are surfacely exposed in the doubly chimeric molecules.

The immunogenicity of HA110–120 CD4 T-cell epitope was assessed *in vitro* by measuring the activation of the LD1-24 T-cell hybridoma (TcH) that recognizes the HA110–120 peptide in association with the I-E^d molecule (Haberman *et al.*, 1990). The half maximal activation of TcH was approximately two log higher than in the case of HA110–120 peptide.

Table 9. Surface exposure of HA110–120 and HA150–159 epitopes in doubly antigenized Ig molecules

	Binding to:			
	Rat anti-mouse IgG2b	Rat anti-mouse Ig/k chain mAb	Mouse anti-HA 150–159 mAb	Rabbit anti-HA 110–120
wild IgG2b	+	+	–	–
IgT,B	+	+	+	+
IgHA-galB	+	+	+	+
T-B peptide	–	–	+	+

The immunogenicity of HA150–159 B cell epitope was studied *in vivo* by immunization of BALB/c mice with various antigens. Immunized mice produced antibodies that bound not only to the HA150–159 peptide but also to the PR8 virus. *Table 10* shows the concentration of anti-HA150–159 antibodies measured 21 days after the immunization of BALB/c mice with 100 μg doubly antigenized Igs or synthetic dipeptide in CFA and with PR8 or B/Lee virus in saline.

Table 10. The anti-HA150–159 peptide antibodies produced by BALB/c mice immunized with various antigens

Immunogen	Concentration of anti-HA150–159 antibodies at day 21 after immunization (μg/ml)
IgT,B	360
IgHA-gal-B	420
T-B synthetic peptide	48
PR8 virus	12
BLee	0

In spite of the fact that influenza virus does not cause a natural infection in mice, the intranasal infection causes a pneumonia subsequent to the multiplication of the virus in the upper respiratory tract and the lungs. Influenza pneumonia in mice is associated with a dramatic loss in weight.

Vaccination of humans or animals against influenza induces the production of antibodies mainly directed against the major surface glycoproteins, namely haemagglutinin (HA) and neuraminidase (NA). Whereas anti-HA antibodies prevent the penetration of the virus into the target cells, anti-NA antibodies inhibit the spreading of the virus. The effect of vaccination is best mirrored in a decrease of virus lung titer subsequent to intranasal or aerosol challenge. The data presented in *Table 11* show the

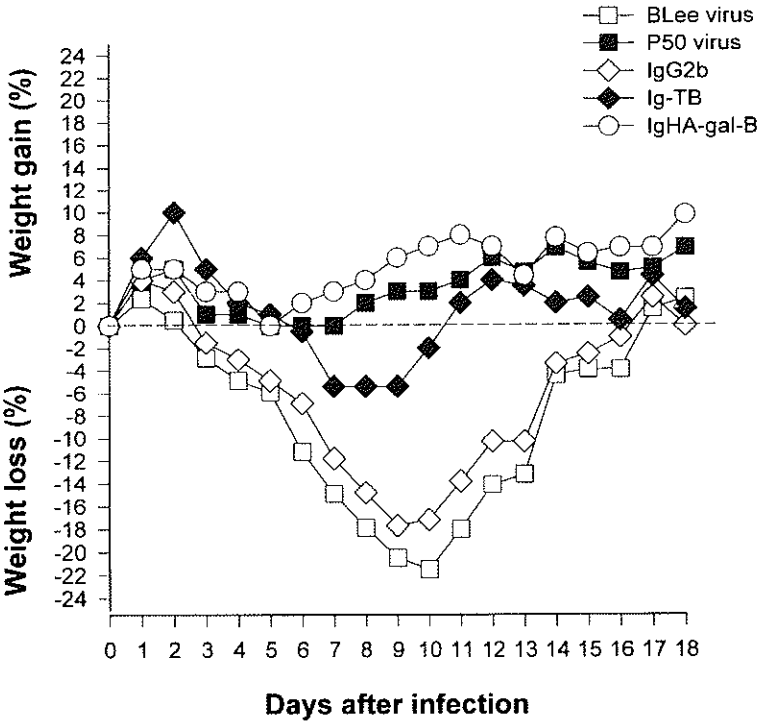

Figure 10. Weight variation after PR8 virus infection of BALB/c mice immunized with various antigens. Groups of seven BALB/c mice each were immunized intraperitoneally with either 5 μg of the P50 reassortant virus or BLee virus control in saline, or with 100 μg, three times at one week interval, of doubly antigenized Ig-TB or Ig-HA-gal-B constructs. Mice were then infected intranasally with two LD50 doses of PR8 virus, and the weight variation per group was daily monitored, up to 18 days post-infection. Similar weight recovery was observed in the case of mice immunized with P50 virus to those immunized with both Ig-TB and Ig-HA-gal-B constructs.

effect of the immunization of BALB/c mice with various antigens on the pulmonary virus titer following the aerosol infection with PR8 virus. Only the mice immunized with P50 virus, a reassortant sharing the HA gene with the PR8 virus, and the mice immunized with IgT,B completely cleared the virus by day 7 following the challenge. The mice immunized with IgHA-gal-B exhibited a drastic reduction of lung titers by day 7, compared to the non-immunized mice. In contrast, the mice injected with IgG2b and B/Lee virus were unable to clear the virus and died by day 16 following the infection.

It is noteworthy that the mice immunized with P50 virus, IgT,B or IgHA-gal-B did not exhibit significant weight loss after the challenge with PR8 virus, compared to those immunized with IgG2b or BLee virus (*Figure 10*).

These data demonstrated that immunization with doubly antigenized molecules elicited immune responses that prevented the development of infuenza pneumonia after the challenge with the PR8 influenza virus. This is probably related to the production of high-affinity anti-HA antibodies. Actually, the injection of mice with high-affinity anti-HA antibodies can protect the SCID mice devoid of mature T- and B-cells against a challenge with a lethal dose of influenza virus (Palladino *et al.* 1995).

Table 11. Pulmonary virus titers of BALB/c mice immunized with various antigens and challenged with PR8 virus

Antigen	Viral titer following the challenge with 1.5×10^4 $TCID_{50}$ of PR8 virus		
	Day 3	Day 7	Day 16
Nil	4.8 ± 0.1[a]	3.4[b]	NS[c]
IgG2b	4.4 ± 0.6	3.1 ± 0.7	NS
IgT.B	4.8 ± 0.7	0	0
IgHA-gal-B	5.5 ± 0.7	1.5 ± 0.5	0
P50 virus	2.1 ± 0.3	0	0
BLee virus	5.1 ± 0.4	3.4 ± 0.5	NS

[a]Pulmonary titre expressed as mean +/-SD of the $\log_{10}TCID_{50}$ of individual titres in groups of at least three mice.
[b]Only one mouse survived at day 7 following the infection.
[c]No survivors.

Casares *et al.* (1997a) have studied the effect of immunization with DNA encoding for the V_H-T,B polypeptide. For this, a plasmid encoding for a variable region of a self Ig in which the CDR2 and CDR3 loops were replaced by the major B-cell epitope (HA150–159) and the CD4 T-cell epitope (HA110–120) of influenza A/PR/8/34 virus under the CMV promoter, was constructed and designated as pV_H-T,B (*Figure 11*). The immune response against PR8 influenza virus was studied in mice immunized three times at three weeks interval with 100 μg pV_H-T,B. Data summarized in *Table 12* demonstrated priming of mice immunized with pVH-T,B plasmid or PR8 virus upon *in vitro* stimulation with HA110–120 peptide. This was correlated to the cytokine production in the cell culture supernatants. Furthermore, these mice mounted an antibody response against both PR8 virus as well as the HA150–159 peptide.

Table 12. Induction of humoral and cellular immune responses in BALB/c mice immunized with pVH-T,B plasmid

Mice immunized with:	Proliferative response (cpm $\times 10^{-3}$)[a]	Interleukin production (pg/ml)		Antibody response (μg/ml) specific for[b]:	
		IFNγ	IL-4	PR8	HA150–159
Nil	622 ± 38	0	0	0	0
Plasmid control	556 ± 42	–	–	–	–
VH-T,B	9321 ± 156	55 ± 3	0	4 ± 2	5 ± 2
B/Lee virus	496 ± 46	0	0	0	0
PR8 virus	13459 ± 352	74 ± 3	24 ± 3	42 ± 9	12 ± 4

[a]T cells from immunized mice were stimulated *in vitro* with PR8 virus and the incorporation of ^{3}H-thymidine was estimated as described by Casares *et al.* (1997).
[b]The concentration of specific antibodies was estimated by RIA, 28 days following the completion of immunization.

It is important to point out that the pattern of cytokine response was strikingly different after immunization with virus compared to pV_H-T,B. Whereas immunization with PR8 virus induced T_H1- and T_H2-like cytokine production, the immunization with pV_H-T,B induced mainly T_H1-like cytokine production (INF-γ). Therefore, the delivery of influenza virus T and B peptides by immunization with naked DNA may represent a genuine model for vaccination. This notion is strongly supported by data showing the clearance of pulmonary virus as well as by the significant survival of mice

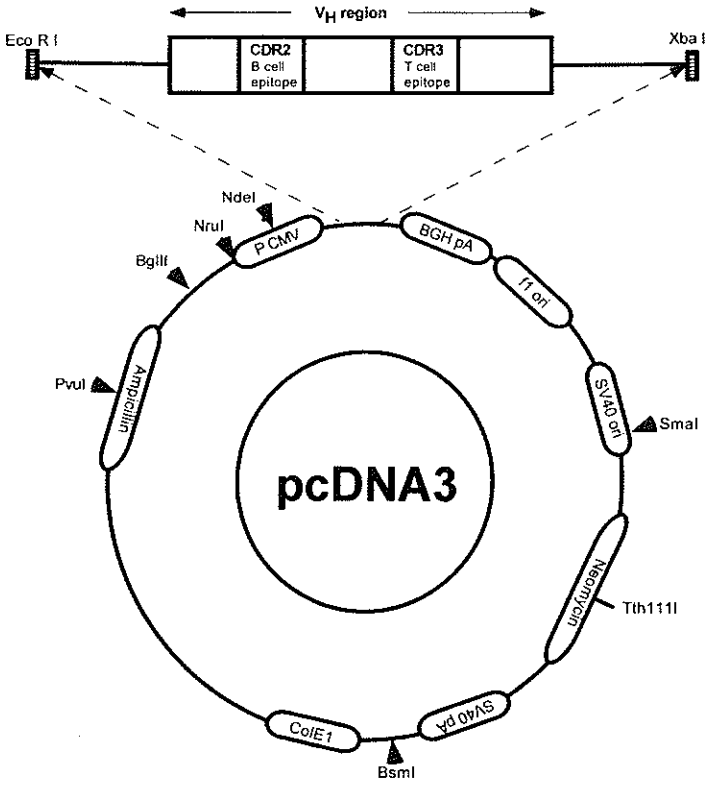

Figure 11. Genetic construction of pV_H-TB plasmid. Generation of the V_H-TB chimeric gene (upper panel) and its insertion in the pcDNA3 plasmid (lower panel).

immunized with V_H-T,B and challenged with lethal doses of PR8 influenza virus (Casares *et al.*, 1997a). Together, these results showed that the immunization with pV_H-T,B induced a host defence reaction comparable with that elicited by the PR8 virus.

ENGINEERED MHC–PEPTIDE CHIMERIC MOLECULES

The major histocompatibility complex (MHC) class I and class II molecules are highly polymorphic glycoproteins that present peptides to the $CD8^+$ and $CD4^+$ T-cells, respectively. The membrane-bound glycoproteins are heterodimers consisting of two non-covalently associated subunits: heavy chain/β-2microglobulin for MHC class I; and α/β chains for MHC class II. A peptide binding-groove is generated by the folding of α1–α2 domains of the MHC class I heavy chain, or the assembly of α1–β1 domains of MHC class II heterodimer (Rothbard and Gefter, 1991). The amino acids residues of the peptide-binding groove are very polymorphic, allowing the presentation of 10^4 different peptides per MHC class I molecule (Cox *et al.*, 1994), and over 2000 different peptides per MHC class II (Hunt *et al.*, 1992; Marrack *et al.*, 1993). MHC class I molecules bind peptides mainly derived from endogenously synthesized proteins (Townsend *et al.*, 1986) whereas MHC class II

molecules bind peptides derived from the processing into lysosomal compartments (Babbit *et al.*, 1985).

The cognate TCR interaction with the MHC–peptide complex can induce either clonal expansion and differentiation, or clonal anergy, depending upon the presence or absence of secondary stimulatory signals (costimulation) provided by professional APCs (Schwartz, 1990). Induction of anergy upon TCR interaction is one of the mechanisms that accounts for the peripheral self tolerance when T-cells recognize tissue-specific peptides presented by non-professional APCs which lack costimulatory molecules. Based on this information, MHC class I and class II molecules loaded with antigenic peptides were considered as platforms for designing immuno-modulatory agents.

Several approaches have been attempted to generate MHC–peptide complexes: affinity-purified MHC molecules extracted from cell membranes or recombinants MHC molecules loaded *in vitro* with specific peptides, genetically engineered, covalently linked peptide/MHC chimeric molecules, or MHC-Ig chimeric molecules.

Natural MHC molecules loaded with peptides

Human and murine MHC class II complexes have been extracted from cell membranes and subjected to peptide elution of natural peptides followed by loading with specific peptides (Jardetzky *et al.*, 1990; Babbit *et al.*, 1985; Nag *et al.*, 1992). Because of the low dissociation constant of bound peptides (Buus *et al.*, 1987; Chen and Parham, 1989) these preparations have a very low yield for peptide loading (0.5% of the protein pool) and still have a high contamination with self peptides.

To increase the yield and purity, the MHC complexes have been dissociated, the monomeric α and β subunits purified and further refolded in the presence of exogenous peptide (Kupinski *et al.*, 1983; Deshpande *et al.*, 1990; Passmore *et al.*, 1992; Rothenhausler *et al.*, 1990; Nag *et al.*, 1993). These natural MHC–peptide complexes were able to induce *in vitro* T-cell apoptosis (Nicolle *et al.*, 1994; Nag *et al.*, 1996) and were effective for treatment of autoimmune diseases in experimental models (Sharma *et al.*, 1991; Spack *et al.*, 1995). However the low yield of peptide loading (5–20% of the starting material), and the risk of contamination with self peptides represent the major concern for the therapeutic use.

Recombinant MHC molecules loaded with peptides

Most of the recombinant MHC molecules described in the literature are truncated molecules lacking the transmembrane/intracytoplasmic domains needed to allow the secretion of soluble assembled heterodimers. Stern and Wiley (1992) reported for the first time that empty HLA-DR1 molecules are secreted by insect cells infected with baculoviruses carrying the α- and β-chain encoding genes. Recombinant MHC class II have also been produced in prokaryotic (Altman *et al.*, 1993; Stockel *et al.*, 1994; Arimilli *et al.*, 1995) and eukaryotic cells (Wettstein *et al.*, 1991; Scheirle *et al.*, 1992; Buelow *et al.*, 1993). After peptide binding, the recombinant MHC complexes were able to interact with specific TCRs and subsequently to stimulate T-cell clones. However, the low association rate of foreign peptides to the empty MHC molecules, and inefficiency in the appropriate folding and dissociation of the MHC heterodimer

after peptide binding procedures (Mottez *et al.*, 1995), led to the development of chimeric molecules able to prevail over some of these inconveniences: covalently linked peptide MHC molecules, tend to generate homogenous populations of MHC–peptide complexes, and single chain MHC molecules, tend to stabilize the heterodimer.

Covalently-linked peptide–MHC chimeric molecules

Kozono *et al.* (1994) constructed soluble murine I-E^{dk}-MCC(91-103) and IA^{d}-OVA(327-339) chimeric molecules in which the peptide was genetically linked to the N-terminus of the β-chain. Insect cells infected with baculovirus encoding for the α and peptide-β genes, secreted correctly assembled heterodimers with the peptide lying in the groove. The covalently linked peptide–MHC complexes were more efficient at activating specific T cell hybridomas than the purified MHC class II heterodimers loaded *in vitro* with the peptides. In addition the I-A^{d}-OVA construct was less stable than the natural counterpart, indicating that the transmembrane/intracytoplasmatic domains may play a role in the stability of the MHC–peptide complexes.

Mottez *et al.* (1995) have generated several MHC class-I chimeric molecules in which a single peptide (Cw3[170–185], A2[170–185] or NP) was linked to the carboxy-terminus of the K^{d} heavy chain. The L-cells transfected with these genetic constructions expressed surface K^{d}-peptide assembled with endogenous β2-microglobulin molecules, and could be lysed by specific CTL clones. The transfected cells were highly immunogenic when injected *in vivo*, as measured by the CTL response or by protection against viral and tumoral challenge.

Single chain MHC heterodimers

To overcome the instability of the MHC heterodimer, single chain genes (SC) encoding for MHC class I heavy chain linked to β2-microglobulin have been constructed (Mottez *et al.*, 1991; Godeau *et al.*, 1992; Mage *et al.*, 1992; Abastado *et al.*, 1993a; Abastado*et al.*, 1993b). Such chimeric molecules exhibited higher stability than the native heterodimer, and bound to similar set of peptides as the native membrane class I molecules (Godeau *et al.*, 1992; Ojcius *et al.*, 1993; Abastado *et al.*, 1993b). While monomeric forms of the single-chain MHC molecules had no effect on T-cell activation, dimers generated by antibody cross-linkage stimulated T-cell to secrete IL-2 (Abastado Iet al., 1995).

Covalently linked peptide single chain MHC molecules

Finally, to obtain homogenous and stable populations of MHC–peptide complexes, single chain MHC molecules with a covalently linked peptide have been generated. While Mottez *et al.* (1995) made a soluble single chain K^{d}-β2m linked to Cw3 peptide (K^{d}-Cw3-β2m), Rhode *et al.* (1996) constructed a single chain I-A^{d} covalently linked to OVA(323–339) peptide. These covalently linked peptide MHC chimeric molecules were efficient in stimulating T-cells. In addition, the T-cell stimulation induced by the MHC class II chimeric molecule was subsequently followed by apoptosis.

It is noteworthy that most of the recombinant MHC molecules described in the literature are monovalent. The affinity of TCRs for the MHC class II–peptide

complexes was estimated at 10^{-5} M, with a half-life of 12 s (Weber *et al.*, 1992; Matsui *et al.*, 1991; Matsui *et al.*, 1994; Seth *et al.*, 1994; Stern *et al.*, 1994; Brown *et al.*, 1993). for the MHC class I molecules the rate off was estimated at 10^{-7} M, and half-time of interaction of 27 s (Corr *et al.*, 1994; Sykulev *et al.*, 1994). Thus, a single TCR/MHC–peptide interaction may not be sufficient to trigger TCR signalling. A longer half-life of the cognate TCR/MHC–peptide interaction can be achieved by cross-linking of multiple TCRs.

Recently, two different forms of physiologically shed soluble HLA (sHLA) molecules have been identified in the sera of patients and healthy individuals (Zavazava and Kronke, 1996): hydrophobic membrane-bound heterodimers and hydrophilic forms that were not detected in the membrane. The latter is a result of the alternative splicing of class I molecules. Cell-membrane sHLA were more efficient than hydrophilic sHLA molecules in inducing apoptosis of primary alloreactive CD8 T cells. This is because the multimers generated through their hydrophobic tails have the ability to cross-link the TCR complexes, and subsequently to trigger early events of apoptosis.

It has been estimated that around 100 interactions may be necessary to activate T-cells (Demotz *et al.*, 1990; Harding *et al.*, 1990). However, Corr *et al.* (1994) suggested that the threshold may require two or more complexes depending on the kinetic characteristics of a particular TCR/MHC–peptide interaction. The latter supposition is supported by Abastado *et al.* (1995), who generated a soluble monovalent single chain-K^d (SC-K^d). When loaded with Cw3 peptide, this chimeric molecule had no effect on the activation of TcH. However, SC-K^d dimerized through the anti-K^d antibody was sufficient to induce Il-2 production followed by a state of T-cell unresponsiveness. The cross-linking of TCRs is essential for effective T-cell activation. This concept was supported by the fact that multimerization of SC-K^d-CW3 complexes through a second antibody induced stronger T-cell response. In addition, tetramers of HLA-A2 loaded with HIV peptides have been obtained by Altman *et al.* (1996) by genetic engineering of a substrate peptide for BirA-dependent biotinylation site linked to the MHC class I heavy chain. A full tetrameric molecule was assembled by folding in the presence of β-2 microglobulin and HIV peptides, following the addition of streptavidin. These molecules have been successfully used to phenotype antigen-specific T-lymphocytes in HIV-infected patients. This indicated a high avidity of the tetramers for the cognate TCRs. However, no effect on specific T-cells has been reported yet for these molecules.

MHC-Ig chimeric molecules

Because T-cell activation requires the interaction of TCRs with a polyvalent array of MHC-peptide complexes, several attempts have been made to genetically engineer divalent forms of MHC molecules stabilized by the Ig Fc segment. A soluble divalent MHC class I (H-$2K^b$) was constructed by Porto *et al.* (1993). This chimeric molecule was generated in such a way that the extracellular domains of the MHC class I-heavy chain were inserted into the variable region of an IgG1-heavy chain. Whereas soluble monomeric forms had no effectively inhibited alloreactive T-cell responses (McCluskey *et al.*, 1988; Arnold *et al.*, 1988; Schneck *et al.*, 1989), the divalent chimeric molecule was able to inhibit the lysis of target cells by specific H-$2K^b$-alloreactive T-cell clones.

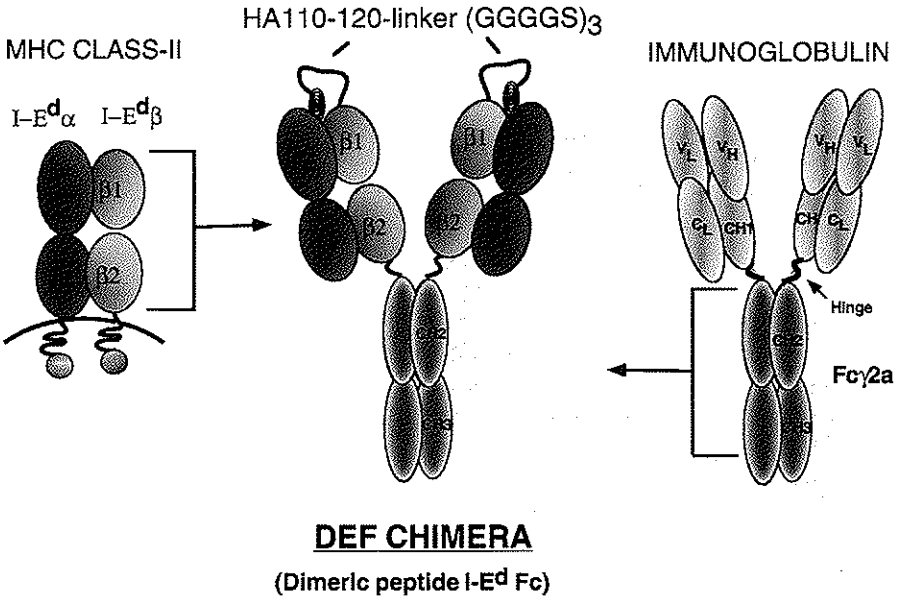

Figure 12. Schematic representation of the recombinant DEF antigen presenting molecule. The murine MHC class II antigen presenting molecule consists of the extracellular domains of I-E$^d\alpha$ and I-E$^d\beta$ chains to which the CD4 T cell immunodominant epitope HA110–120 of the haemagglutinin (HA) of the A/PR/8/34 influenza virus was covalently linked at the N-terminus of the I-E$^d\beta$ chain. The HA110–120/I-E$^d\alpha\beta$ complex was dimerized by the Fc portion of an IgG2a linkied at the C-terminus of the I-E$^d\beta$ chain.

Recently, Lepley *et al.* (1997) have constructed divalent MHC class I molecules composed of the extracellular domains of H-2L(d) and the Fc portion of IgG1 or IgM. When loaded with peptides, the divalent H-2L(d)-Fcγ1 efficiently inhibited the response of specific class-I restricted T-cells *in vitro*.

Covalently linked peptide MHC-Ig chimeric molecules

To obtain a homogenous population of divalent MHC–peptide complexes, a divalent form of a covalently linked peptide MHC class II molecule has been genetically engineered. The chimeric molecule consists of the extracellular domains of I-E$^d\alpha$ and I-E$^d\beta$ chains to which the CD4 T-cell immunodominant epitope HA110–120 of the haemagglutinin (HA) of the A/PR/8/34 influenza virus was linked at the N-terminus of the I-E$^d\beta$ chain (Casares *et al.*, 1997b). The HA110–120/I-E$^d\alpha\beta$ complex was dimerized by the Fc portion of an IgG2a linked at the C-terminus of the I-E$^d\beta$ chain. SF9 insect cells infected with baculovirus carrying both I-E$^d\alpha$ and HA110–120/I-E$^d\beta$/Fcγ2a genes, secreted a disulphide stabilized dimer of the HA110–120/I-E$^d\alpha\beta$/Fcγ2a molecule, designated as DEF (*Figure 12*).

The DEF molecule preserved the structural and biochemical characteristics of both MHC–peptide complex and Fcγ2a portion (*Figure 13*), and it was able to:

(i) bind specifically and with high avidity to the cognate TCR. Whereas the affinity of the TCR for HA110–120-IE^d molecule was estimated at 10^{-5} M

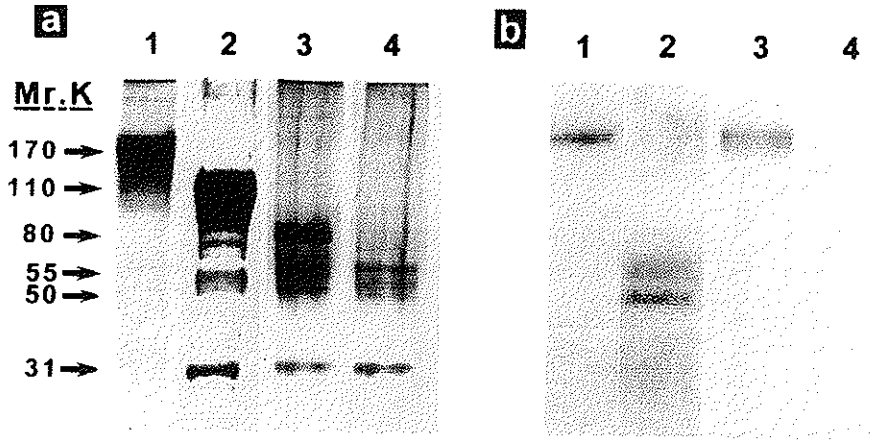

Figure 13. SDS-PAGE and Western blot analyses of the recombinant DEF antigen presenting molecule. SDS-PAGE was performed on 4–12% polyacrylamide gradient gels, according to the standard procedure of Laemli. *Panel a*, represents DEF molecule, as revealed by silver stain: lane 1, no reduction and no boiling; lane 2, no reduction with boiling; lane 3, reduction without boiling; lane 4, reduction with boiling. *Panel b* represents the Western blot analysis after transferring the gels onto PVDF membranes. Lane 1 represents DEF molecule under no reducing/no boiling conditions, and lane 2, DEF molecule under reducing and boiling conditions, as revealed with ^{125}I-goat anti-mouse γ2a Abs. Lane 3, represents DEF molecule under no reducing/no boiling conditions, and lane 4, DEF molecule under reducing/boiling conditions, as revealed with ^{125}I-14-4-4 mAb.

(Weber *et al.*, 1992), the phenotyping of antigen-specific T-cells by DEF molecule (*Figure 13*) suggest a similar affinity with that of rat anti-TCR clonotypic antibody (6.5.2) mAb.

(ii) bind to the FcγRII receptors (FcR);

(iii) mediate cytotoxicity by activation of the complement cascade. It was reported for the first time that a chimeric molecule expressing the Ig-Fc portion preserves this Ig-associated biological function.

(iv) trigger early production of IL-2 in cognate T-cells (*Figure 14*).

The strategy of designing antigen presenting molecules as stable dimeric forms of MHC class II–peptide complexes may represent a novel platform for the development of prodrugs endowed with T-cell immunomodulatory effects.

Conclusions

Synthetic peptides display poor immunological activity *in vivo* due to a low efficiency in generation of MHC–peptide complexes on relevant APCs. This is caused by the extracellular peptidases that shorten the half-life of the peptides and by the limited access of the synthetic peptides to the specialized cellular compartments of APC, where the generation of MHC–peptide complexes occurs in an optimal manner.

Engineering self molecules carrying peptides may circumvent these inconveniences because of their resistance to extracellular proteolysis and their efficient internalization into the endosomes of APC. The chimeric self molecules bearing foreign peptides can be either genetically or enzymatically engineered. Chimeric Igs can be also engineered as encoding plasmids. Igs are effective vehicles of self origin.

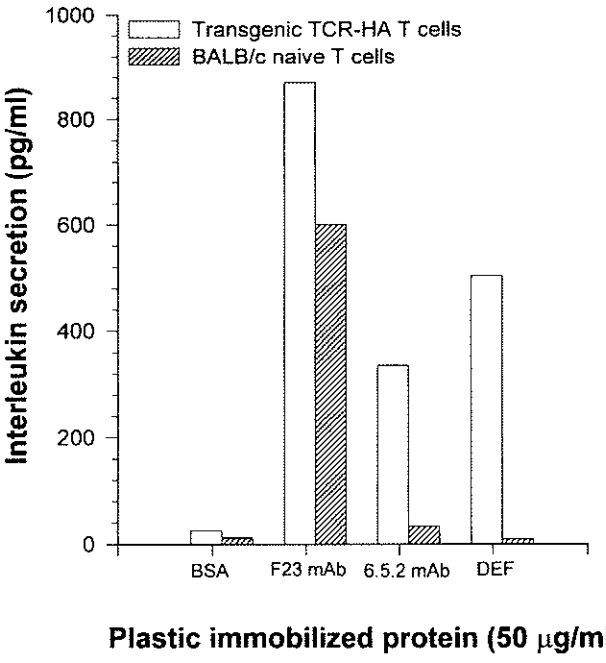

Figure 14. Induction of IL-2 from cognate T cells by the recombinant DEF antigen presenting molecule. Purified T cells from spleens of HA110–120-TCR transgenic mice or naive BALB/c mice (2×10^5) were incubated for 24 h in polystyrene tubes coated with 50 μg/ml BSA, F23 mAb (anti-TCR Vβ8), 6.5.2 anti-TCR clonotypic mAb, or DEF, in the absence of APCs. The production of IL-2 was determined in the cell culture supernatants by ELISA. The pg/ml values represent the mean of triplicate wells.

While Ig-bearing T-helper epitopes are effective immunogens upon exogenous administration, Ig chimeras carrying CTL epitopes are immunogenic only if administered as encoding plasmids or plasmid-transfected cells. In addition, some enzymatically engineered Ig-peptide and genetically engineered MHC–peptide chimeric molecules exhibit their immunomodulatory effects by circumventing the intracellular processing requirements, and even participation of professional APCs.

Compared with foreign proteins of microbial origin, self molecules bearing foreign peptides still have two major drawbacks: (i) limited activity throughout outbred populations due to the haplotype restriction, and (ii) lower protective ability due to the lack of multiple synergistic epitopes. The former can be overcome to a certain extent by engineering multiple T- and B-cell epitopes on the same carrier.

On the other hand, self molecules bearing foreign peptides have as their major advantage over the native foreign proteins, that the antibody response to irrelevant epitopes of the carrier is minimal.

Self molecules that bear antagonist peptides may efficiently interfere *in vivo* with ongoing autoimmune responses. MHC–peptide chimeric molecules, by their intrinsic ability to engage cognate TCRs, may display modulatory effects on T-cells. Particularly, engagement of TCRs in the absence costimulatory signals may lead to anergy of the autoreactive T-cells.

In conclusion, an efficient way to manipulate the immune response against micro-

bial or autoreactive epitopes is to attach the epitopes by various means to carriers of self origin.

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