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Reverse immunogenetic and polyepitopic approaches for the induction of cell-mediated immunity against bovine viral pathogens

Nagendra R. Hegde[1] and S. Srikumaran[2]*

[1]*Department of Molecular Microbiology and Immunology, L220, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201–3098 and*
[2]*Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583–0905, USA*

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Abstract

The control of several infectious diseases of animals by vaccination is perhaps the most outstanding accomplishment of veterinary medicine in the last century. Even the eradication of some pathogens is in sight, at least in some parts of the world. However, infectious diseases continue to cost millions of dollars to the livestock industry. One of the reasons for the failure to control certain pathogens is the limited emphasis placed on cell-mediated immunity (CMI) in the design of vaccines against these pathogens. Traditionally, vaccine-induced immunity has been studied in relation to antibody-mediated protection. More recent studies, however, have focused on understanding CMI and developing means of inducing CMI. This review focuses on recent advances made in the study of CMI in general and of cytotoxic T lymphocytes in particular. Parallels from studies in human and mouse immunology are drawn in order to point out implications to bovine immunology, specifically for immunity against bovine herpesvirus 1.

Introduction

Our ability to combat cancer and infectious diseases in humans and animals has made great strides during the last few decades, although several diseases and syndromes remain elusive [e.g. acquired immunodeficiency syndrome in humans and bovine respiratory disease complex (shipping fever) in cattle]. Advances in the field of prophylaxis against intracellular pathogens and tumors have resulted in the emergence of a unique discipline, termed 'vaccinology', which encompasses the design and production of pharmaceutical and biological products. Early efforts in vaccine development focused mainly on the protective ability of the product, while more recent studies have included the analysis of the mechanism(s) of protection induced by the vaccines.

*Corresponding author: Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583–0905, USA. E-mail: ssrikumaran1@unl.edu

Immune response

The immune system is involved in maintaining homeostasis by surveillance, through the differentiation of self from non-self and the elimination of foreign bodies. The immune response can be broadly divided into two major components: the innate response, which is non-specific, and the acquired response, which is specific to the antigen eliciting the response. Each has a humoral and a cell-mediated component. In general, multiple arms of the immune system meet the challenge of responding to a few or several antigens to eliminate or contain a pathogen. An ideal vaccine should stimulate both humoral and cell-mediated immunity.

The antigen-specific humoral response is mediated by antibodies. Historically, more emphasis has been placed on the antibody response to vaccines that have played a pivotal role in the global or regional eradication or containment of several infectious diseases. A few examples of such diseases are measles, mumps, polio, smallpox, rinderpest and pseudorabies.

Antibodies function by binding directly to antigens

and neutralizing the infectious agent. Neutralization of viruses may be at any of the various steps involved in the replication and biogenesis of viral particles within an infected cell (Dimmock, 1993). Alternatively, antibodies eliminate infectious agents by (i) opsonizing organisms or infected cells and sensitizing them to lysis mediated through the complement pathway, or (ii) opsonizing infected cells and sensitizing them to killing mediated by cells that carry receptors for antibodies (antibody-dependent cell-mediated cytotoxicity, or ADCC) (Goldsby *et al.*, 2000). One or all of these functions may be operative against a pathogen. However, because antibodies are unable to enter cells, they cannot completely eliminate obligate intracellular pathogens such as viruses. Once a pathogen enters and replicates within a cell, its clearance requires either ADCC or the involvement of antigen-specific CMI. However, ADCC is a relatively inefficient process as the effector function of antigen-specific CMI requires far fewer relevant functional molecules than ADCC to trigger an immunological response (Oldstone, 1994). Thus, antigen-specific CMI plays an important role in the containment of intracellular pathogens. The involvement of antigen-specific CMI is critical in the case of viruses that have the capacity to spread from cell to cell without being released into the extracellular space and consequently into the body fluids, such as blood, as in the case of the alphaherpesviruses (Lodmell *et al.*, 1973). Different aspects of antigen-specific CMI are currently being elucidated, and the study of the contribution of CMI to immunity after vaccinations is still in its infancy.

The principal element of antigen-specific CMI is the lysis of cells harboring an intracellular pathogen by T lymphocytes. T cells are functionally divided into T helper (T_H) cells and cytotoxic T lymphocytes (CTLs), which generally express the specific cell-surface molecules CD4 and CD8 respectively. Unlike antibodies, which recognize antigens in their entirety, T cells recognize antigen in the form of short peptides presented by molecules encoded by the genes of the major histocompatibility complex (MHC). The recognition unit of T cells consists of a heterodimeric T-cell receptor (TCR). The unique interaction between TCRs and MHC molecules defines self and distinguishes self from non-self or foreign molecules. The rules of MHC restriction govern the cognitive specificity of $CD8^+$ and $CD4^+$ T lymphocytes towards MHC class I and class II molecules respectively (Rothbard and Gefter, 1991). The TCR–MHC interaction thus provides the pivotal control switch for antigen-specific CMI.

MHC molecules

The MHC is a large, tightly linked complex of genes that were identified by the pronounced effect of the gene products on the immune response to transplants. Subsequent studies identified three classes of molecules, designated I, II and III, encoded by the MHC gene complex. The MHC molecules that regulate antigen-specific humoral immunity and CMI are encoded by genes in the class I and class II clusters. Genes in both of these clusters encode molecules that are highly polymorphic but have similar domain organization (Trowsdale, 1995). For example, three loci encode human MHC class I products, and each locus contains several different alleles that are expressed in a given population. The alleles encoded by these tightly linked loci are inherited as two sets, one from each parent, and each set of alleles is known as a haplotype.

Each MHC allele encodes a protein (allomorph) that forms a heterotrimer along with an MHC- or non-MHC-encoded protein and a linear peptide. The structure of MHC molecules, as revealed by X-ray crystallography, consists of distinct domains characterized by the presence of an intradomain disulfide bond. The MHC class II molecules are made up of polypeptides α and β, each of which has a constant and a variable domain. The polymorphic domains $\alpha 1$ and $\beta 1$ together form a membrane-distal groove that accommodates a peptide of 12–25 amino acids. On the other hand, MHC class I molecules consist of a polymorphic heavy (H) or α chain and a non-polymorphic light chain, β_2-microglobulin (β_2m). The former is anchored to the membrane while the latter non-covalently associates with the H chain. Two ($\alpha 1$ and $\alpha 2$) of the three domains of the H chain form the membrane-distal peptide-binding groove that accommodates a peptide of 8–10 amino acids (Madden, 1995). For both class I and class II molecules, the two polypeptides as well as the short peptide are necessary for the stable expression of the trimolecular complex on the cell surface under physiological conditions.

Antigen processing and presentation

Antigen processing and presentation can be defined as the process by which a self or a foreign protein is converted by a series of concerted steps into peptides capable of associating with MHC molecules and being expressed on the surface of cells to produce a functional complex that is capable of being recognized by T cells (Ziegler and Unanue, 1981). The presentation and subsequent recognition of the antigenic peptide trigger an appropriate response by T cells specific to that antigen. The stimulated T cells proliferate (lymphoproliferation) and perform effector functions, including the secretion of cytokines, providing help to B cells, and killing cells expressing the foreign antigen. A responding T cell thus recognizes a foreign peptide in association with a self MHC molecule. The myriad antigens presented to T cells can be functionally divided into endogenous (derived from within the cell) and exogenous (derived from outside the cell), depending on their origin and the

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pathway of presentation. The two pathways are generally used by class I and class II MHC molecules respectively (Hosken *et al.*, 1989).

Antigen processing and presentation by MHC class II molecules (exogenous pathway)

The MHC class II molecules, in general, present antigenic peptides to T_H lymphocytes. They are expressed constitutively by 'professional' antigen-presenting cells (macrophages, B cells and dendritic cells) that primarily present antigens internalized from the extracellular milieu. The processing of extracellular antigens and presentation by MHC class II molecules is referred to as the exogenous antigen presentation pathway, and is illustrated in Figure 1. The antigens that enter the endosomal/lysosomal compartment, by phagocytosis or receptor-mediated endocytosis, are degraded to minimal peptides by acid-dependent proteases. The class II α and β chains are cotranslationally translocated into the endoplasmic reticulum (ER), and folded with the assistance of 'chaperones'. The loading of class II molecules with peptides in the ER is precluded by the binding of the invariant chain (Ii), which forms a complex with the α and β chains and targets the complex to the endosomal/lysosomal compartment. The Ii is then degraded except for an oligopeptide known as the class II-associated Ii peptide (CLIP), the region of Ii that is bound in the peptide-binding groove. The DM molecules assist enzymatically in the removal of CLIP and the subsequent loading of cognate peptides, in a specialized class II loading compartment called MIIC. The function of DM is regulated by DO molecules. Once loaded with the peptide, the class II molecules are transported through the vesicular transport pathway and expressed on the cell surface (Pieters, 1997; Vogt *et al.*, 1997; Weeink and Gautam, 1997; Jensen, 1998). Other proteins, such as the members of the tetraspan family and the heat-shock protein family, may be involved during the generation of peptides and the assembly of the class II molecules in lysosomes and MIIC respectively (Hammond *et al.*, 1998; Panjwani *et al.*, 1999).

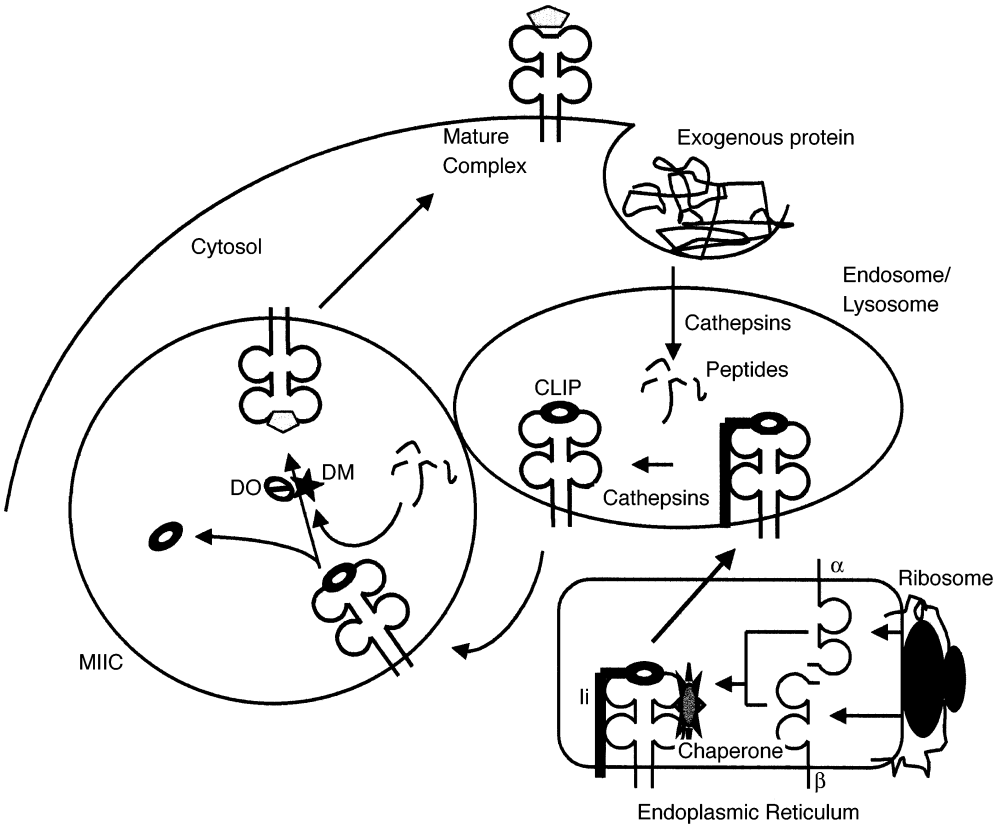

Fig. 1. The exogenous pathway of antigen presentation. The co-translationally translocated MHC class II α and β chains fold partially, with the assistance of chaperones, in the endoplasmic reticulum. The αβ heterodimer immediately associates with the invariant chain (Ii), which targets the whole complex to the endosomes/lysosomes. Partial degradation of Ii by cathepsins leaves an unstable αβ heterodimer bound with the class II-associated Ii peptide (CLIP). The antigen obtained by endocytosis/phagocytosis is degraded by cathepsins in the endosomes/lysosomes into smaller peptides. The CLIP is removed and the peptides are loaded onto class II molecules in a specialized MHC class II loading compartment (MIIC) by the enzymatic function of HLA-DM molecules. The activity of HLA-DM molecules is regulated by HLA-DO molecules. The mature, stable, trimeric class II molecule presents antigen to $CD4^+$ T-lymphocytes on the cell surface.

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Antigen processing and presentation by MHC class I molecules (endogenous pathway)

The processing of proteins synthesized in or introduced into the cytoplasm and the presentation of the resulting peptides by the MHC class I molecules to CTLs is referred to as the endogenous antigen presentation pathway (Fig. 2). The endogenous pathway is better understood than the exogenous pathway and will be described in greater detail here because of its direct relevance to the generation of antigenic peptides for CTL response. The MHC class I molecules are expressed at different levels by all the nucleated cells in the body (Singer and McGuire, 1990). The class I H chain and β_2m are cotranslationally translocated into the lumen of the ER. The newly synthesized H chain is bound immediately by calnexin, an ER membrane chaperone, which helps in the folding of the H chain and the assembly of the class I heterodimer. Binding of β_2m displaces calnexin and further stabilizes the H chain. Subsequently, the dimer is joined by calreticulin, an ER luminal chaperone. Complexes of the H chain with calnexin or calreticulin are joined by ER60, an ER luminal protein, which assists in the formation of the intradomain disulfide bond (Williams and Watts, 1995; Lehner and Trowsdale, 1998; Cresswell *et al.*, 1999). Unassembled class I molecules are shunted out of the ER into the cytosol, deglycosylated and degraded (Hughes *et al.*, 1997).

The assembled class I molecules present peptides originating from proteins synthesized in or introduced directly into the cytoplasm. These proteins include constitutively expressed cellular proteins and those from replicating pathogens or arising from cellular dysregulation (e.g. tumors). The proteins are generally degraded by the proteasomes, the major cytoplasmic endoproteases outside the lysosomes, after ubiquitination of the target proteins (Ciechanover, 1994; Rock *et al.*, 1994). The generation of peptides may be altered qualitatively by interferon-γ (IFN-γ) secreted into local environments during an immune response (Tanaka *et al.*, 1997). The peptides are transported into the ER, in the presence of ATP, by the heterodimer transporter associated with antigen processing (TAP) (Momburg and Hammerling, 1998), which consists of TAP1 and TAP2 subunits.

The loading of the peptides onto class I molecules occurs in a complex involving TAP, calreticulin, ER60 and tapasin (Lehner and Trowsdale, 1998). The exact

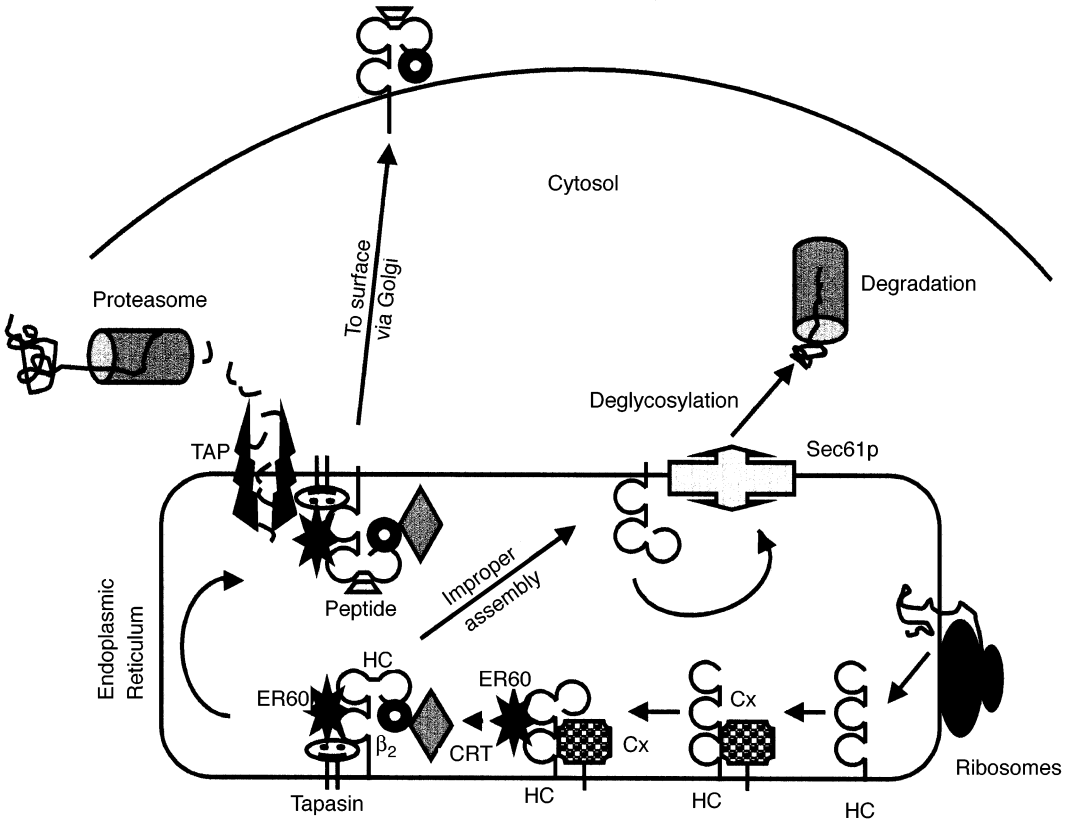

Fig. 2. The endogenous pathway of antigen presentation. The co-translationally translocated MHC class I heavy chain (HC) folds partially, with the assistance of calnexin (Cx) followed by calreticulin (CRT), β_2m and ER60, in the endoplasmic reticulum (ER). The peptide (the third component of the trimolecular MHC complex) is generated by the degradation of the intracellularly derived antigen by the proteasomes. The peptide is relayed to the ER by the transporters associated with antigen processing and presentation (TAP). The complete folding of the MHC molecule occurs with the assistance of the peptide-loading complex, comprising calreticulin, ER60, tapasin and TAP. The folded class I molecule matures through the Golgi complex and presents the antigenic peptide on the surface of the cell for surveillance by the cytotoxic T lymphocytes. Improperly folded class I molecules are shunted out of the ER by the Sec61p complex, deglycosylated in the cytosol and degraded by the proteasomes.

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function of ER60 here is unknown, but it may trim class I-bound peptides (Lindquist *et al.*, 1998). Tapasin stabilizes the TAP molecules and holds the assembly complex together (Grandea *et al.*, 1997; Lehner *et al.*, 1998). Each TAP1 subunit binds four molecules of tapasin, each of which binds one class I heterodimer (Ortmann *et al.*, 1997). While the N-terminal domain of tapasin interacts with calreticulin and ER60 and is required for the stable association of calreticulin and MHC class I, the C-terminal domain binds TAP (Bangia *et al.*, 1999). A fraction of the class I allomorphs may also be loaded independently of TAP, through the direct capture of peptides generated by the action of the signal peptidase on proteins that are translocated into the ER (Wei and Cresswell, 1992).

The trimolecular complex (H–β_2m–peptide) egresses out of the ER, and translocates to the cell membrane via the secretory pathway. As few as three to five trimolecular complexes carrying the same antigenic peptide are enough to elicit a peptide-specific CTL response (Sykulev *et al.*, 1996). The interaction between the TCR and MHC class I molecule is very short-lived, and is strengthened by the binding of the CD8 molecule to the α3 domain of the H chain (Garcia *et al.*, 1996).

The degeneracy and specificity of peptide binding to MHC class I molecules

Since only three or four loci encode class I molecules and the MHC molecules are codominantly expressed, each individual of a species can express a maximum of six to eight different class I molecules, if the individual is heterozygous at all the loci. Therefore, the myriad intracellular peptide antigens clearly outnumber the class I molecules expressed by an individual. It is therefore necessary for each class I allomorph to bind and present several thousands of peptides. Thus, each class I allomorph binds peptides degenerately and yet maintains the specificity of the immune response to a particular MHC–peptide complex (Engelhard, 1994).

Crystal structure of MHC class I molecules

The X-ray crystallographic analysis of human and murine class I molecules (Madden, 1995) indicates that the general structure is the same for a class I molecule irrespective of whether the peptide-binding groove contains a self or a foreign peptide (Madden *et al.*, 1993). The α3 domain folds with β_2m and supports the underside of the peptide-binding groove formed distal to the membrane by the α1 and α2 domains. The floor of the groove is formed by eight β-pleated sheets, and the walls are formed by α-helices. The peptide binds in the groove in an extended conformation with restricted polarity, and the groove generally accommodates a peptide of eight to ten residues. The floor of the binding groove accommodates six pockets that are characteristic of each allomorph, and the side-chains of the amino acids of the peptide protrude into these pockets. While certain pockets can only accommodate certain amino acid side-chains, others are more flexible (Bjorkman *et al.*, 1987a, b; Saper *et al.*, 1991; Fremont *et al.*, 1992). The more restricted pockets are responsible for the specific interactions with the allomorph, and the flexible pockets allow for the degeneracy of peptide binding (Madden, 1995).

Peptide binding to MHC molecules is not the final step of an immunological phenomenon. Through the TCR, the T cell recognizes the antigenic peptide displayed by the MHC molecule. The TCR consists of α and β chains, each of which has a membrane-proximal constant domain and a membrane-distal variable domain. The antigen-binding site of the TCR consists of polypeptide loops of the variable domains that are contributed by both the α and the β chain (Davis and Bjorkman, 1988). Binding of different peptides to the MHC molecule introduces differential conformational and charge display, mostly in the middle of the MHC–peptide complex. The TCR recognizes the structural and ionic heterogeneity in the peptide, along with small but detectable conformational differences in the MHC (Madden, 1995; Bjorkman, 1997).

Allele-specific peptide motifs

While MHC polymorphism increases the chance that at least one peptide from any intracellular pathogen encountered by members of a species is bound by at least one MHC class I molecule, each individual expresses only a small number of different class I molecules. Thus, each class I isoform must bind a finite, yet large, number (thought to be 2000–10 000) (Hunt *et al.*, 1992; DiBrino *et al.*, 1993) of peptides that are structurally diverse. Therefore, the group of heterogeneous peptides bound by each class I allelic product should have some commonality. One or more positions in the sequence of the array of peptides bound by an allomorph carry the same or chemically similar amino acids. The side-chains of these amino acids protrude into the corresponding pockets in the peptide-binding groove and anchor the peptide in the groove. Hence these amino acid residues are called anchor residues. These anchor residues and their relative positions in the peptide sequence constitute the 'allele-specific peptide motif' (ASPM) of that particular allomorph (Rammensee *et al.*, 1995). The chemical nature and the location of the anchor residues are closely related to the composition, structure and accessibility of the pockets in the peptide-binding groove of the MHC molecule (Saper *et al.*, 1991). While the anchor residues control the MHC-specific binding, the non-anchor residues contribute to the variability in the peptides. Therefore, MHC class I molecules are peptide receptors of stringent specificity, and yet

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match the diversity of the T-cell repertoire by degenerate peptide binding.

Because the stable expression of class I molecules requires the peptide component (Ljunggren *et al.*, 1990), the class I molecules expressed by normal somatic cells bind peptides derived from self proteins that are constitutively turned over. These bound peptides can be isolated by mild acid treatment (Wallny and Rammensee, 1990) of affinity-purified single class I allomorphs (a species-specific anti-class I antibody is used if single allelic transfectants are available. Alternatively, an allele-specific monoclonal antibody is employed in the isolation of a particular class I allomorph). The pool of peptides is a comprehensive sample of peptides associated with that particular class I allomorph (Hunt *et al.*, 1992; Huczko *et al.*, 1993). The peptide pool can then be sequenced as it is (pool sequencing) or after high-resolution chromatographic separation (Falk *et al.*, 1991; DiBrino *et al.*, 1993). The ASPM is then identified on the assumption that the location of residues constituting the motif is the same in relation to the N-terminus for all the peptides extracted from the same MHC allomorph (Engelhard, 1994).

Identification of CTL epitopes

The major component of MHC-restricted antigen-specific CMI against intracellular pathogens involves CTL-mediated immunity. The CTLs eliminate infected cells by contact-dependent lytic mechanisms (Hahn *et al.*, 1994; Morreta, 1997). Alternatively, CTLs inhibit the replication of intracellular pathogens by the secretion of cytokines such as IFN-γ and tumor necrosis factor α (Guidotti *et al.*, 1999a, b).

The minimal sequence that elicits an immune response is known as an epitope. For an antibody, the sequence could represent a peptide, a saccharide or an oligonucleotide. For a T cell, the epitope is represented by an unmodified or a modified peptide sequence.

The conventional method of identification of CTL epitopes for intracellular pathogens (illustrated in Fig. 3) begins with the growth and culture of CTLs recognizing target cells infected with the pathogen. Further characterization of antigen specificity usually requires the cloning of individual antigen-specific CTLs (unless only one or two epitopes dominate the CTL response to a

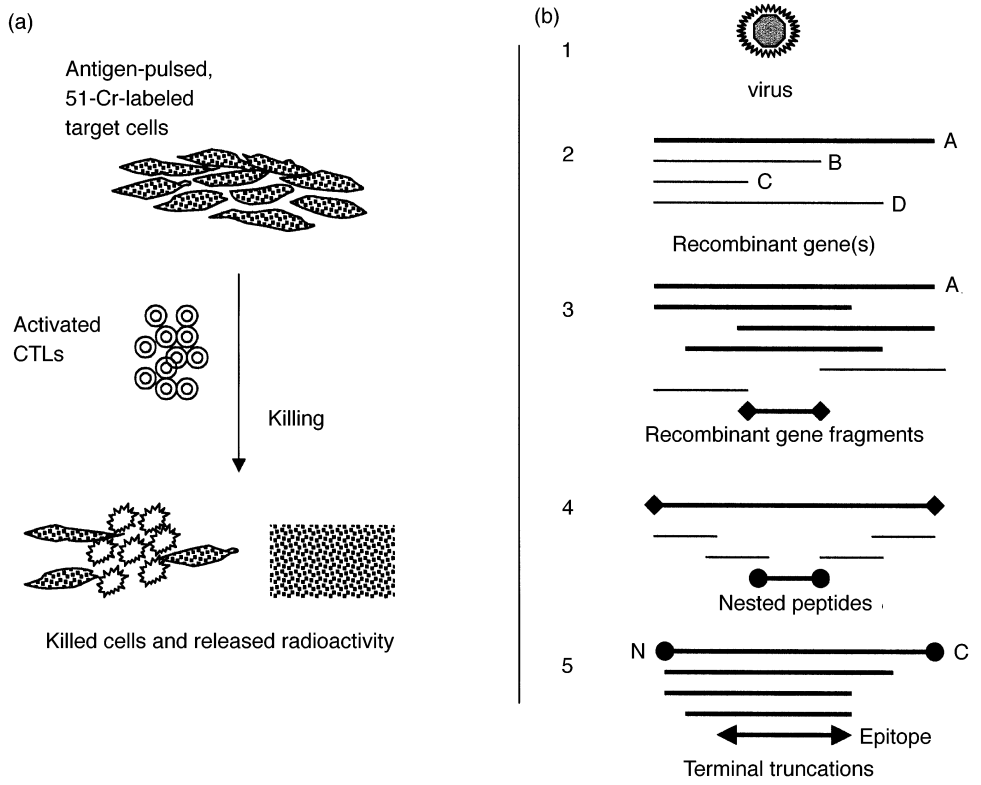

Fig. 3. Schematic representation of the conventional method of identification of cytotoxic T-lymphocyte (CTL) epitopes. (a) CTL-mediated killing is commonly assessed by the microcytotoxicity assay, in which target cells expressing the antigen are labeled with radioactive chromium and CTLs are added at various effector-to-target ratios. The release of radioactive chromium over background levels indicates lysis of target cells. (b) The CTL epitope is identified in a series of steps using the microcytotoxicity assay. The intracellular pathogen, which is known to elicit a CTL response (step 1), is dissected by using recombinant DNA technology and synthetic peptides. Thick lines indicate target protein sequences eliciting a CTL response. The protein (indicated as A) targeted by the CTLs is identified by cloning the genes (A, B, C, D) into a vector (plasmid or virus) and expressing it in the cells known to be targets for CTL-mediated killing (step 2). The region within the protein A that elicits the CTL response is narrowed down by using vectors expressing smaller fragments of the protein A (step 3). The epitope is then identified by the use of overlapping (step 4) and truncated (step 5) synthetic peptides representing the sequence of the smaller region of the protein that sensitizes the target cells for lysis by the CTLs.

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pathogen). The antigen specificity is characterized by the use of target cells expressing individual genes of the pathogen. The expression of individual genes is achieved by cloning the DNA constituting the gene(s) into and infecting or transfecting the target cells with recombinant plasmid or viral vectors respectively. Once the individual protein specificity is identified, recombinant vectors are constructed so that fragments of the protein can be expressed. Such recombinant vectors containing truncated genes encoding fragments of the protein are then tested for the sensitization of target cells for CTL-mediated lysis, in order to localize approximately the epitope to a region of the protein. Further localization within the fragment is performed by testing nested synthetic peptides consisting of the sequence of the antigenic fragment used in sensitizing the targets for lysis by the CTLs. Finally, the minimal epitope is identified by the use of peptides with N- and C-terminal truncations of the longer peptide that is recognized by the CTLs (Townsend *et al.*, 1986; Hanke *et al.*, 1991).

The identification of CTL epitopes by the conventional method is laborious. The ASPMs provide a relatively easy method to accomplish this objective. The identification of CTL epitopes by the prediction of candidate epitopes based on ASPMs is known as the 'reverse immunogenetic method', and is illustrated in Figure 4. Because the ASPMs are the same whether the peptides bound by MHC molecules are self or foreign, CTL epitopes conform to the same rules of MHC binding as the normal cellular peptides. Therefore, it is possible to predict 8- to 10-mer peptides from proteins of intracellular pathogens by applying the ASPM along with the length restriction and the proteolytic cleavage specificities (Gaddum *et al.*, 1996b; Hegde and Srikumaran, 1996). Because synthetic peptides can define epitopes directly by sensitizing target cells to CTL-mediated effector functions (Townsend *et al.*, 1986), peptides representing candidate epitopes can be synthesized and tested for their ability to serve as epitopes recognized by bulk or cloned CTLs. Whereas only a few CTL epitopes had been identified before the advent of the ASPMs, several epitopes on viruses, tumor antigens, intracellular parasites and other proteins have been identified since (Pamer *et al.*, 1991; Banks *et al.*, 1993). One of the strengths of this strategy is the identification of subdominant and conserved epitopes (Wentworth *et al.*, 1996; van der Most *et al.*, 1998).

Bovine MHC molecules

The MHC molecules in cattle are known as bovine lymphocyte antigens (BoLA). The MHC locus of cattle is divided into class I, class IIa, class IIb and class III

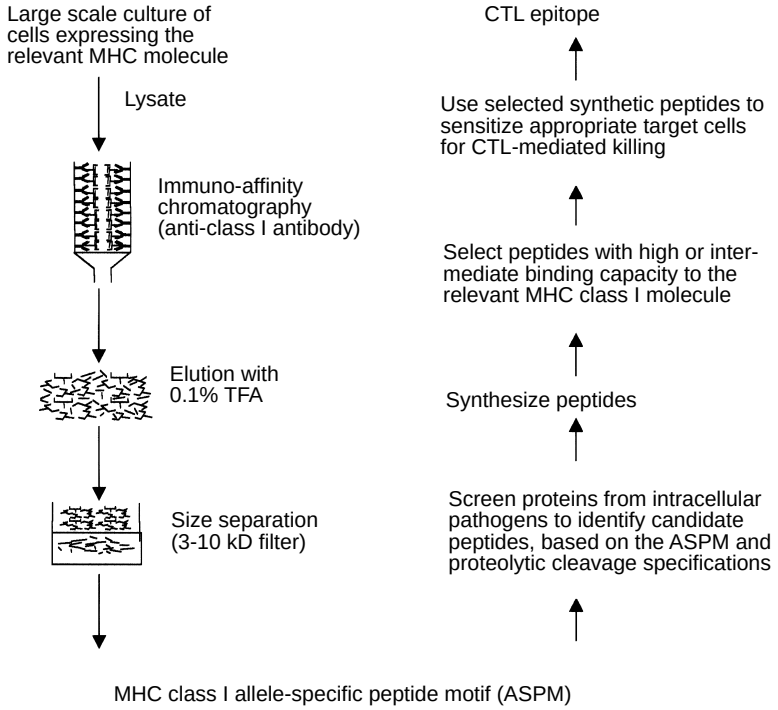

Fig. 4. Schematic representation of the reverse immunogenetic method of identification of CTL epitopes. The reverse immunogenetic method utilizes the common characteristics of peptides bound to MHC class I molecules, namely allele-specific peptide motifs (ASPMs; see text for details). The ASPMs are identified by affinity-purifying class I molecules, eluting the associated peptides by mild acid treatment, and determining the quantity of different amino acids at each position of the peptide. The information obtained is applied to the sequence of known proteins from intracellular pathogens to identify candidate CTL epitopes. Synthetic peptides representing such candidates are used directly in CTL assays (as shown in Fig. 3) to identify the epitope.

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regions. The class I and IIa regions encode proteins homologous to human and murine MHC class I and II molecules respectively. The function of proteins encoded in the class IIb region is not known (Lewin, 1996).

Bovine MHC class I molecules appear to be similar to human class I molecules, in that (i) several anti-human class I monoclonal antibodies cross-react with the respective bovine molecules (Brodsky and Parham, 1982), and (ii) charged anchor residues are found in ASPMs of cattle (Bamford *et al.*, 1995) and humans but not those of mice (Rammensee *et al.*, 1995). Serologically, more than 50 BoLA class I specificities have been defined (Davies *et al.*, 1994), with distinct phenotypic frequencies in different breeds of cattle (Bull *et al.*, 1989). Based on DNA sequence, transcript and biochemical analyses, the presence of at least three class I loci has been inferred, and there is evidence that two or three loci are expressed by individual animals (Ellis *et al.*, 1992; Joosten *et al.*, 1992; Al-Murrani *et al.*, 1994; Garber *et al.*, 1994). However, the expression pattern is complicated, because most of the haplotypes seem to be encoded by a single locus and different haplotypes express a variable array of class I genes, none of which seem to be expressed consistently in association with a particular haplotype (Ellis and Ballingall, 1999).

Antigen-specific CMI in cattle

The relationship of some of the BoLA class II molecules to susceptibility or resistance to diseases has been studied, most notably in the bovine leukemia virus system (Lewin, 1996). The function of BoLA class II molecules in presenting antigens to T_H cells has been demonstrated in the case of ovalbumin (Rothel *et al.*, 1990), *Brucella abortus* (Smith *et al.*, 1990), *Mycobacterium bovis* (Pollock *et al.*, 1995), *Theileria* spp. (Glass and Spooner, 1991; Baldwin *et al.*, 1987), *Babesia* spp. (Brown *et al.*, 1998a; Stich *et al.*, 1999), *Anaplasma marginale* (Brown *et al.*, 1998b), *Cowdria ruminantium* (Totte *et al.*, 1999), *Cryptosporidium parvum* (De Graaf *et al.*, 1998), *Fasciola hepatica* (Brown *et al.*, 1994), bovine leukemia virus (Mager *et al.*, 1994), bovine viral diarrhea virus (BVDV) (Rhodes *et al.*, 1999), foot and mouth disease virus (Collen, 1994) and bovine herpesvirus 1 (BHV-1) (Tikoo *et al.*, 1995). Minimal T_H epitopes have been identified in some of these cases (Collen, 1994; Mager *et al.*, 1994; Pollock *et al.*, 1995).

The function of BoLA class I molecules in immunity to infectious agents is less well understood. Cell-mediated cytotoxicity was first demonstrated in cattle for *Theileria parva* (Eugui and Emery, 1981). To date, CTLs have been well characterized for *T. parva*. In the case of this parasite, protection is mediated mainly by lymphocytes rather than antibodies, as revealed by adoptive transfer experiments. Additionally, the mechanism involved is MHC class I-restricted lysis of infected cells by $CD8^+$ lymphocytes. The clearance of the pathogen *in vivo* correlates directly with the increase in the number of cytolytic cells in the blood, during both primary and secondary infections (Morrison and McKeever, 1998). Class I-restricted proliferative or cytolytic $CD8^+$ lymphocytes have also been demonstrated in the case of BHV-1 (Denis *et al.*, 1993), bovine respiratory syncytial virus (BRSV) (Gaddum *et al.*, 1996a), bovine leukemia virus (Gatei *et al.*, 1993), BVDV (Beer *et al.*, 1997), foot and mouth disease virus (Childerstone *et al.*, 1999), *Brucella abortus* (Splitter *et al.*, 1996) and *Theileria annulata* (Preston *et al.*, 1983). A recent study identified a protective CTL epitope on bovine leukemia virus in sheep (Hislop *et al.*, 1998). However, not even a single BoLA class I-restricted CTL epitope has been identified for any pathogen of cattle.

CMI to bovine herpesvirus 1

BHV-1 is an important causative agent of bovine respiratory disease (BRD) complex, which costs over US$500 million annually in direct losses to the US cattle industry alone (United States Department of Agriculture, 1996). The virus primarily infects the respiratory and genital tracts of cattle, causing infectious rhinotracheitis and infectious pustular vulvovaginitis and balanoposthitis respectively. It is also associated with keratoconjunctivitis, dermatitis and meningoencephalitis (Gibbs and Rweyemamu, 1977). The other viral pathogens involved in the BRD complex are BVDV, BRSV, bovine adenovirus and parainfluenza virus type 3 (Rosenquist, 1983). Infection with one or more of these agents is followed by immunosuppression and secondary bacterial invasion, often leading to fatal pneumonia. BRD is manifested as a synergy between the above viruses and the bacteria *Pasteurella haemolytica, P. multocida, Haemophilus somnus, H. influenzae, Mycoplasma* spp. and/or *Chlamydia* spp. (Babiuk *et al.*, 1988).

Vaccination programs are in place to combat BRD complex. The available vaccines include several products for the primary viral pathogens, including BHV-1, and the secondary bacterial pathogens. For BHV-1, both inactivated and modified live virus (MLV) vaccines are in use. The inactivated vaccines are unsatisfactory in that they do not induce long-lasting humoral immunity and fail to induce CMI. MLV vaccines of BHV-1 may fail to induce herd immunity despite protecting animals at the individual level. Like the wild-type virus, the MLV strains can also cause disease and immunosuppression, and undergo latency and reactivation (Frerichs *et al.*, 1982; Whetstone *et al.*, 1986; Nataraj *et al.*, 1997). Therefore, an ideal vaccine against BHV-1 must elicit an immune response that (i) neutralizes the virus at the portal of entry, (ii) eliminates infected cells by cytotoxicity or contains the virus in infected cells by inhibition of

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replication of the virus, (iii) controls the establishment of latency and/or reactivation from latency, (iv) does not produce immunosuppression, (v) is compatible with pregnancy, and (vi) allows infected animals to be differentiated from vaccinates (van Drunnen Little-van den Hurk *et al.*, 1993; van Oirschot *et al.*, 1996). However, the development of such a vaccine in the near future is unlikely.

Two of the mechanisms by which the virus causes immunosuppression are the induction of apoptotic death of several types of leukocytes (Hanon *et al.*, 1998), including $CD4^+$ T cells (Eskra and Splitter, 1997; Winkler *et al.*, 1999) and the down-regulation of MHC class I molecules (Hinkley *et al.*, 1998; Nataraj *et al.*, 1997). Together, these may lead to defective CTL priming. Therefore, there is a need to design MLV vaccines that do not carry the component of the virus that causes immunosuppression, or to design recombinant vaccines that contain genes encoding protective antibody and CTL epitopes. While humoral immunity to BHV-1 has been well studied (van Drunnen Little-van den Hurk *et al.*, 1993) and several antibody epitopes have been mapped in cattle (Marshall *et al.*, 1988; van Drunnen Little-van den Hurk *et al.*, 1990), the contribution of CMI during BHV-1 primary infection, reactivation from latency, and reinfection is not well understood. As regards the identification of CTL epitopes, not even a single bovine CTL epitope of BHV-1 has been mapped.

CMI may play an important role in primary BHV-1 infections (Babiuk *et al.*, 1988b). However, most of the studies of antigen-specific CMI to BHV-1 have concentrated on lymphoproliferation rather than the CTL-mediated lysis of infected target cells. Several BHV-1 proteins induce lymphoproliferation (Hutchings *et al.*, 1990), and T_H epitopes have been localized on the glycoproteins gC (Leary and Splitter, 1990) and gD (Tikoo *et al.*, 1995). The proliferative response to BHV-1, however, may not correlate with the level of specific antibodies, or protection against challenge with the virulent virus (Hutchings *et al.*, 1990; Wentink *et al.*, 1990).

MHC-restricted anti-BHV-1 cytolytic responses have been demonstrated in short-term cultures (Campos and Rossi, 1986a, b). The derivation of BoLA-Aw6/11-restricted BHV-1-specific CTL clones has been reported (Splitter *et al.*, 1988), but the antigen-specificity of these clones was not evaluated. Incidentally, the majority of the bovine × murine T-cell hybridomas developed in our laboratory to study the CMI to BHV-1 were MHC class II- restricted (Nataraj and Srikumaran, 1994). Recently, the major glycoproteins have been identified as the targets for bovine CTLs (Denis *et al.*, 1993). A study in our laboratory identified three CTL epitopes of BHV-1 in the murine model (Zatechka *et al.*, 1998). Thus, while both T_H and murine CTL epitopes have been identified, the bovine CTL response to BHV-1 has not been well characterized.

Use of the reverse immunogenetic method to identify bovine CTL epitopes

The identification of ASPMs in cattle has been hampered by the unavailability of allele-specific antibodies and by the lack of cloned class I genes. Recently, several bovine MHC class I genes have been cloned and transfected into heterologous cell types (Toye *et al.*, 1990; Sawhney *et al.*, 1995; Ellis *et al.*, 1996). We and others have used these cells for the identification of four ASPMs in cattle (Hegde *et al.*, 1995; Gaddum *et al.*, 1996c). Another study used homozygous lymphocytes to identify the ASPM of BoLA-A20 (Bamford *et al.*, 1995). These ASPMs (Table 1) can be used to predict epitopes from intracellular pathogens (Table 2).

As regards the reverse immunogenetic approach for the identification of CTL epitopes in cattle, the ASPMs of only five class I allomorphs have been identified so far (Table 1). By using the ASPM of BoLA-A11, we identified groups of candidate peptides on glycoproteins gC and gD that sensitized target cells for lysis by bovine CTLs (Hegde *et al.*, 1999), but we were unable to propagate the CTL cultures long enough to identify the individual epitopes. The ASPMs of more bovine class I allomorphs need to be identified. However, these studies are hindered by the low number of class I genes (fewer than 30) that have been cloned and sequenced. Furthermore, there is a need for defined systems of generation of, and assay for, CTLs *in vitro*. For example, in the human system, B lymphoblastoid cell lines suitable for use as antigen-presenting cells and target cells in cytotoxicity assays can be developed easily by transformation of B lymphocytes with Epstein–Barr virus. In the murine system, several MHC-typed B-cell lines and macrophage

Table 1. Allele-specific peptide motifs of BoLA class I molecules

Allele	Position 2	Position 3	Position 7	Position 9	Position 10
BoLA-A11	Proline			Isoleucine/valine	
BoLA-A20	Lysine			Arginine	
BoLA-HD1		Isoleucine			
BoLA-HD6	Glutamine				
BoLA-HD7	Valine		Isoleucine		Tyrosine

The positions indicate the location of the amino acid relative to the amino terminus of the peptide. In the case of BoLA-A11, the peptide can be nine or ten amino acids long.

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Table 2. Examples of candidate CTL epitopes from bovine intracellular pathogens predicted from the ASPMs of BoLA-A11, -A20 and -HD7

ASPM	Peptide sequence	Source
BoLA-A11	372 PPTEALLFL	Bovine herpesvirus type 1 α-transinducing factor
BoLA-A11	307 APSTGVYEL	Bovine coronavirus E2 glycoprotein precursor
BoLA-A11	90 APTGPGVVV	Bovine rotavirus outer capsid protein VP4
BoLA-A20	39 NKRGDRSAR	Bovine papillomavirus coat protein VP2
BoLA-A20	828 SKLFLSLTR	Bovine adenovirus type 3 fiber protein
BoLA-A20	384 GKCRLEACR	Rinderpest virus hemagglutinin-neuraminidase
BoLA-A20	472 IKTEQRNIR	Bovine parainfluneza 3 virus
BoLA-A20	203 PKSNERLDR	Bovine herpesvirus 1 tegument protein
BoLA-A20	210 PKVNNHDCR	Bovine respiratory syncytial virus fusion protein
BoLA-HD7	72 SQVWYFLIIGY	Bovine papillomavirus small t antigen
BoLA-HD7	295 SAVAIFCITYY	Vaccinia virus hemagglutinin precursor

The numbers before the peptide sequences indicate the position of the first amino acid in the protein specified. The list is not exhaustive.

cell lines are readily available. A similar situation does not exist in cattle. We have recently described a method for obtaining autologous cell lines from MHC-typed animals (Hegde *et al.*, 1998), and these cells have been used as targets in CTL assays (Hegde *et al.*, 1999). Retroviral vectors have been used successfully to deliver antigens to high endothelial cells (Earnest-DeYoung *et al.*, 1999), but their use as targets in CTL-mediated killing has not been evaluated. The availability of more MHC-typed cells should help advance our knowledge of the CTL response to pathogens of cattle.

Recent trends in strategies for CTL-based vaccination

Vectors containing single genes encoding known antigens can be used as immunogens. However, these recombinant single-gene vaccines have several disadvantages: (i) because the MHC molecules are highly polymorphic, the gene of interest may lack an adequate number of CTL epitopes to cover the MHC diversity of the target population; (ii) the targeted protein may show antigenic variation leading to escape variants; (iii) in cases where the pathogen in question causes immunosuppression, the targeted antigen might be the immunosuppressive protein, theoretically making the recombinant protein immunosuppressive; (iv) the target antigen may be downregulated, leading to immune evasion (e.g. in melanoma) (Jager *et al.*, 1997); and (v) unwanted responses may be elicited (e.g. antibodies to the protein may enhance infectivity, as in the case of Dengue, human immunodeficiency and other viral infections) (Dimmock, 1993). Some or all of these problems may be circumvented by the use of epitope-based vaccines. Identification of CTL epitopes is a prerequisite for the development of such vaccines.

Synthetic peptides with proper adjuvants can elicit CTL responses (Schulz *et al.*, 1991; Feltkamp *et al.*, 1993; Lipford *et al.*, 1995). However, they may be unstable or form secondary structures, and require additional standardization as well as toxicity testing for the adjuvant. Furthermore, CTLs induced by peptides may be of low affinity (Carbone *et al.*, 1988). Therefore, an alternative strategy is to include epitopes as minigenes, i.e. as small fragments of DNA just sufficient to encode an epitope (Whitton *et al.*, 1993), and to use the DNA construct for immunization purposes (Suhrbier, 1997). Single nonameric peptide epitopes encoded by minigenes not only elicit an MHC-restricted, antigen-specific CTL response but also protect hosts from lethal challenge with the relevant pathogen (del Val *et al.*, 1991; Harty and Bevan, 1992; Ciernik *et al.*, 1996). Multiple minigenes such as these, inserted into the vector either tandemly or linked by spacers (the 'string-of-beads' strategy), are biologically relevant (Whitton *et al.*, 1993) irrespective of whether the epitopes are from the same pathogen (Whitton *et al.*, 1993; Thomson *et al.*, 1995, 1996, 1998b; Toes *et al.*, 1997). Long-lived memory responses can be elicited both by this strategy and by using a lipopeptide vaccine (Livingston *et al.*, 1997; Ishioka *et al.*, 1999). Additionally, vectors carrying minigenes encoding different epitopes restricted by different MHC class I or class II molecules elicit CTL or T_H/antibody responses, respectively, to all the epitopes in the context of the relevant MHC molecules, indicating that there is no interference between (i) responses to different epitopes and (ii) presentation by different MHC molecules (Thomson *et al.*, 1995, 1996, 1998a). More significantly, relevant epitopes arranged in tandem in the same vector can elicit CTL, T_H and antibody responses (An and Whitton, 1997). Therefore, this string-of-beads strategy can elicit relevant immune responses to multiple CTL, T_H and/or antibody epitopes from single or multiple pathogens restricted by single or multiple class I or class II molecules.

One of the limitations of the string-of-beads approach is the construction of the vector to include a number of epitopes restricted by several MHC allomorphs to encompass the majority of the population. However, peptide binding studies involving several human MHC class I molecules have recently revealed the existence of MHC 'supertypes', which are defined by their association

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with the same or chemically similar ASPMs, called 'supermotifs' (Sidney *et al.*, 1996; Sette and Sidney, 1998). While single allotypes predominate only in certain subpopulations, each supertype can cover 40–50% of any ethnic population. A combination of as few as four supertypes can cover more than 90% of any ethnic population (Sidney *et al.*, 1996). The supertypes, which were originally identified by binding studies alone, have now been validated biologically in the case of *Plasmodium falciparum* (Doolan *et al.*, 1997), hepatitis B virus (Bertoni *et al.*, 1997), human immunodeficiency virus (Threlkeld *et al.*, 1997), Epstein–Barr virus (Khanna *et al.*, 1998), hepatitis C virus (Chang *et al.*, 1999), melanoma (Kawashima *et al.*, 1998) and epithelial tumors (Kawashima *et al.*, 1999). The A2 and B7 supertypes from humans and the repertoire of CTLs restricted by them have now been extended to chimpanzees (Bertoni *et al.*, 1998).

Recently, MHC class II-restricted supertypes have been proposed based on the chemical composition of the class II peptide binding groove (Ou *et al.*, 1997, 1998), and functional evidence of their existence has been demonstrated in previous studies (Ou *et al.*, 1992; Martinez-Soria *et al.*, 1994; Zelisaewski *et al.*, 1994; Chauhan *et al.*, 1996). However, validating MHC class II supertypes is complicated by the fact that class II molecules present antigens quite promiscuously.

The combination of supertype and string-of-beads strategies should lead to effective CTL-based vaccines to combat important intracellular pathogens. A fascinating possibility is the introduction of multiple epitopes from the same or different pathogen(s) but restricted by class I molecules from different species to elicit relevant CTL responses (Hanke *et al.*, 1998; Hanke and McMichael, 1999). This strategy should be very useful in the case of zoonotic diseases, and would (i) contain the spread of viruses and intracellular parasites to or from carrier hosts, and (ii) reduce the costs of controlling intermediate hosts and vectors, especially in the case of arthropod-borne diseases.

Concluding remarks

Until recently, the efficacy of vaccines has been evaluated mainly on the basis of protection mediated by antibodies. While there is no doubt that antibodies have played, and continue to play, a major role in vaccine-induced immunity, the contribution of CMI to immune responses to the same vaccines has been largely ignored. Current investigations favor a role for CMI, along with antibodies, in the response to vaccination. If the recent studies with recombinant minigene vaccines is any indication, the future of antiviral vaccines may rest on these novel strategies of construction of vaccines based on both humoral and cell-mediated immunity. In cattle, studies involving more MHC class I genes should lead to the identification of more ASPMs and possibly supermotifs. Standardization with regard to the generation and function of CTLs in relation to bovine viral diseases is required in parallel. These steps should pave the way for the identification of CTL epitopes for incorporation into vectors as minigenes to efficiently elicit CTL responses in cattle.

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