A Human Dendritic Cell–Based Method to Identify CD4+ T-Cell Epitopes in Potential Protein Allergens

Marcia Stickler, Jeanette Mucha, David Estell, Scott Power, and Fiona Harding

Genencor International, Palo Alto, California, USA

We developed an assay to determine the location of immunodominant CD4+ T-cell epitopes in any protein. The method uses CD4+ T cells from community donors in conjunction with dendritic cells derived *in vitro*. Synthetic peptides constructed to describe the sequence of the protein of interest are cocultured with dendritic cells and CD4+ T cells, and T-cell proliferation is measured. Data are compiled over a large replicate of human donors to pinpoint immunodominant, usually promiscuous epitope regions. We have applied this technique to a known food allergen, the Brazil nut 2S storage globulin protein, and to two potential food allergens, the Cry1Ab and Cry3Aa proteins. We show epitope data for these three proteins. This assay can be used as a tool to guide the selection and qualification of future potential food transgenes. *Key words*: dendritic cells, food allergens, human donors, T-cell epitopes. *Environ Health Perspect* 111:251–254 (2003). [Online 21 January 2003]
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For the immune system to respond to a protein with a finely tuned, high-affinity antibody response, antigen-specific CD4+ helper T cells must be activated. Activation of CD4+ T cells is a prerequisite for differentiation along either the Th1 or Th2 pathway. Activated Th2 cells are strongly associated with the advent of allergy (Kapsenberg et al. 1998; Mazzarella et al. 2000; O'Hehir et al. 1991; Van Neerven et al. 1996). Activation of T cells requires the recognition via their T-cell receptors of linear peptide antigens presented in the context of a cell-surface human leukoctye antigen (HLA) class II molecule. Any given protein immunogen will contain a discrete number of epitope regions capable of inducing activation for a particular HLA class II allele. Interestingly, the same T-cell epitopes are capable of inducing either Th1- or Th2-type responses (Van Neerven et al. 1994), depending on various environmental and antigen-presenting cell–specific factors (Constant and Bottomly 1997; Lanzavecchia and Sallusto 2001). There is considerable interest in the description of T-cell epitopes because the inclusion of helper epitopes improves the immune response to synthetic vaccine constructs (Alexander et al. 1994, 1998; del Guercio et al. 1997). Peptide epitopes have been used in the treatment of immunologic disorders such as allergy (Oldfield et al. 2001; Rolland et al. 2000) and cancer (Kobayashi et al. 2000; Slansky et al. 2000). Finally, manipulation of commonly promiscuous T-cell epitopes can be used to create reduced-immunogenicity proteins for use in a variety of applications (Warmerdam et al. 2002a, 2002b).

There are currently methods to determine peptide binding to some HLA class II-DR and -DQ molecules. Some of these methods measure the relative strength of the peptide–HLA interaction using isolated class II molecules and purified peptides. Other methods predict peptide binding to HLA using computer algorithms (Fleckenstein et al. 1999; Sturniolo et al. 1999; Yu et al. 2002). These predictive methods are more successful for class I molecules because of the more rigid requirements for peptide length and anchor residues. Unfortunately, peptide binding to HLA is not sufficient to predict the presence of a functional interaction with T cells. There are many examples in the literature of poorly binding T-cell epitopes and of tightly binding peptides that are not T-cell epitopes (Adorini et al. 1988; Fugger et al. 1996; Lo-Man et al. 1998; Ma et al. 1999; Velazquez et al. 2001). In addition, amino acids that flank the epitope core have been shown to have profound effects on T-cell activation in the absence of any effects on HLA binding (Godkin et al. 2001).

We have developed a functional assay that localizes T-cell proliferative responses to peptide epitopes using human community donor cells as the test material (Stickler et al. 2000). The localization of epitopes in our assay is based on a population approach, in that a large replicate of community donor responses is compiled and analyzed for the presence of an "immunodominant" peptide. Interestingly, many HLA class II alleles present similar epitopes ("promiscuous" T-cell epitopes), a property caused by shared binding pockets among the multitude of HLA class II alleles (Southwood et al. 1998). We have noted HLA-DR associations with particular peptide responses (Stickler et al. 2000) but assume that our assay largely identifies promiscuous HLA-DR supertype–associated epitopes.

Our assay was developed to predict functional T-cell epitopes in a population of individuals who have not been previously exposed to the protein under study. This is important for two reasons. First, many novel therapeutic and recombinant proteins are environmentally "new" in that human exposure to these proteins is not detected; therefore, methods are needed to predict *a priori* immunogenicity. Second, evidence from the literature suggests that as an immune response develops, T-cell epitope complexity increases. T-cell epitopes that "prime" the system can be completely discrete from secondary epitopes that arise as the response matures (Muraro et al. 2000). This is especially pronounced when studying T-cell clones, where an individual cloned line may have strict specificity for an epitope that is not an immunodominant epitope as defined by a pooled T-cell analysis. In human population–derived data where there is a known sensitization rate [e.g., the ~1% of the general population who are verified sensitive to peanuts (Sicherer and Sampson 2000)], our results likely expose both the immunodominant and the subdominant epitope regions in the protein of interest. Although not shown here, immunodominant and secondary epitopes could be distinguished in the described assay if the input CD4+ T cells were separated into naive (CD4+ CD45RO−) and memory (CD4+ CD45RO+) populations.

We have applied this assay system to the study of a known food allergen, Brazil nut 2S storage protein Ber e 1. We also tested two bacterial endotoxins known to induce in occupationally exposed workers antigen-specific antibodies (Bernstein et al. 1999) that have been associated with hypersensitivity reactions in humans (Ferber 1999; Netting 2000). Our results suggest that differences in allergenic potential can be determined using this assay.

Materials and Methods

Protein sequences. The mature 2S storage protein from *Bertholletia excelsazil* (Brazil nut) consists of two small polypeptides, a light chain of 64 amino acids and a heavy chain of 82 amino acids (accession no. P04403). *Bacillus thuringiensis* (*Bt*) Cry1Ab, the protoxin sequence from *Bt* serovar *aizawai*, consists of a

single 610-amino-acid polypeptide (accession no. A26461). Cry3Aa, the protoxin sequence from *Bt* subspecies *san-diego*, consists of a 644-amino-acid polypeptide (accession no. P07130). Mature protein sequences were used to design peptides. Signal sequences and other prosequences were not included.

Peptides. All peptides were purchased from Mimotopes (San Diego, CA, USA) as multipin syntheses (PepSets) (Maeji et al. 1990). Peptides were resuspended in dimethyl sulfoxide (Sigma, St. Louis, MO) at 1 or 2 mg/mL, and stored frozen at –70°C until use. Peptides were synthesized as 15-mers that overlapped by 12 amino acids. The sequence of both the light and the heavy chain of Ber e 1 were used to create a 27-peptide-containing set. The sequence of the light chain is encompassed in the first six amino acids of the set, and the heavy chain in the final 21 peptides. The sequences of Cry1Ab and Cry3Aa were tested as sets of 200 and 211 peptides, respectively.

Peptide sets for the two *Bt* proteins were very large. To facilitate testing, these sets were tested as pools of two consecutive peptides per well.

Donor pool. All human blood samples were obtained from the Stanford University Blood Center (Palo Alto, CA) or the Sacramento Medical Foundation (Sacramento, CA). Each donor was HLA haplotyped for DRβ1 and DQβ using a low-stringency sequence-specific primer-polymerase chain reaction (SSP-PCR) kit from Bio-Synthesis (Louisville, TX). The HLA-DR and -DQ expression by our donor pool was found to be not significantly different from a North American reference standard (Mori et al. 1997; data not shown). Our donor pool does show evidence of slight enrichments for ethnicities common to the San Francisco Bay area.

Epitope mapping assay. The assay has been described in detail elsewhere (Stickler et al. 2000). Briefly, dendritic cells were differentiated *in vitro* from blood monocytes largely according to published protocols (Zhou and Tedder 1996) but with a few modifications, including the addition of interleukin-1α and tumor necrosis factor-α on day 5 of culture. Purified CD4+ T cells from the dendritic cell donor were cocultured with dendritic cells and approximately 5 μg/mL of peptide for 5 days. Each peptide was tested at least in duplicate. For small peptide sets, such as Ber e 1, the peptides were routinely tested in triplicate. After 5 days, proliferation was assessed by tritiated thymidine incorporation. Results for each peptide were averaged. A stimulation index (SI) for each peptide was calculated by dividing the average proliferation for a given peptide by the average proliferation of control wells containing dendritic cells, CD4+ T cells, and dimethyl sulfoxide in the absence of peptide.

Data analysis of the pooled *Bt* peptide sets was based on replication of responses in neighboring wells rather than between two separate replicates for each peptide.

Data analysis. A response to a peptide was tabulated as positive if the SI was > 2.95. SI values for each donor were compiled for each peptide set, and the percentages of responders are reported. The average background response rate for each peptide set was calculated by averaging the percentage response for all of the peptides in the set. Major epitopes are defined as having a percentage response that is 3-fold or more than the background. Statistical significance was calculated using Poisson statistics for the number of responders to each peptide within the data set. The response to a peptide was considered significant if the number of donors responding to the peptide was different from the Poisson distribution defined by the data set with a $p < 0.05$.

HLA associations. HLA-DR and -DQ types were analyzed for associations with responses to defined epitope peptides. We used a chi-squared analysis with one degree of freedom. Where an allele was present in both the responder and nonresponder pools, a relative risk was calculated.

Results

Epitope map of Brazil nut 2S storage protein.
The epitope mapping assay was performed on a donor set of 92 community blood bank donors using peptides describing the heavy and light chains of Ber e 1 (Figure 1A). The light chain of Ber e 1 was described by peptides 1–6. The heavy chain was described by peptides 7–27. The overall background response to the peptides in this set was 4.27%. This background level is higher than our overall epitope mapping average (average of ~3% over ~20 proteins tested; data not shown). We have observed that background responses are higher when testing peptide sets derived from proteins with known human exposure rates (data not shown). One epitope (peptide 4 corresponding to amino acids 10–24) was found in the light-chain sequence that met the criterion of 3-fold the background response to this peptide set, returning a total percent of responders equal to 18.48%. Three other prominent regions were found in the heavy-chain protein at peptide 12 [amino acids 16–30 (9.78%)], peptide 16 [amino acids 28–42 (7.61%)], and peptide 20 [amino acids 40–55 (11.96%)] that encompass a nested set of epitopes in the heavy-chain polypeptide. The percentage of responses to epitopes 16–30 and 40–55 are less than 3-fold (12.8%) but are more than 2-fold the background rate. The third epitope (28–42) does not reach 2-fold the background. Figure 1B shows the distribution of responses within the Ber e 1 data set. Peptide 4 had the most responses tabulated, a total of 17 out of 92 tested donors. There were 11 responses to peptide 20. This response rate to peptide 4 was highly significant, with $p < 0.0001$. The response to peptide 20 was also significant, with $p = 0.02$.

HLA associations within the Ber e 1 data set. The HLA-DR and -DQ types of donors who responded to peptides 4 (light-chain amino acids 10–24) and 20 (heavy-chain amino acids 40–55) were analyzed for the presence of any statistically significant enrichment in HLA subtypes. HLA-DQ2 was significantly associated with a nonresponse to peptide 4 ($p = 0.05$, relative risk = 0.29). There was a positive association between the presence of HLA-DR15(2) and a response to peptide 20 ($p = 0.04$, relative risk = 3.0). Interestingly, the strongest association found was a positive association between a response to peptide 12 and the presence of HLA-DR13 ($p = 0.01$, relative risk = 4.44).

Epitope map of Cry1Ab. The epitope mapping assay was performed on a donor set
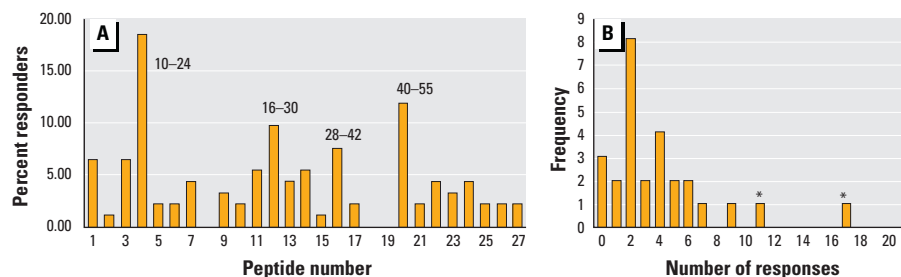

Figure 1. Mapping and designation of epitopes in Ber e 1. (*A*) We tested 92 community donors for reactivity with 27 15-mer peptides derived from the sequence of the Ber e 1 heavy and light chains. The peptides overlapped by 12 amino acids. Individual donor responses that reached an SI of > 2.95 were tabulated as positive. The percentage of the 92-donor pool that responded to each peptide is on the y-axis. The linear peptide designation is indicated on the x-axis. The location of each prominent peptide is indicated by numbers, which correspond to the amino acids of the light- and heavy-chain linear sequences. (*B*) Distribution of responses to peptides. The number of donor responses (x-axis) is plotted versus the frequency (y-axis). One peptide in the Ber e 1 data set accumulated 17 responses (peptide 4), and one accumulated 12 responses (peptide 20).
*Statistically significant responses.

of 48 community blood bank donors using pooled peptides describing the sequence of *Bt* Cry1Ab (Figure 2A). Many peptides were associated with a 0% donor response. On the advice of our statistician, we calculated the background response and determined epitopes using data only from the peptides that returned responses. The background rate for the peptide set was determined to be 2.95%. This background response is consistent with our overall average and suggests that our donor population is not exposed to this protein at levels that could result in an immune response. One epitope meeting the 3-fold cutoff value was detected at peptide 145 [amino acids 433–447 (10.4%)]. A second prominent epitope region was noted at amino acid positions 76–90 (8.33%), but it does not meet the 3-fold cutoff. Figure 2B shows the distribution of responses to the Cry1Ab peptide set. Only one response rate was statistically significant, at peptide 145, with *p* = 0.006. HLA associations were not determined because of the low number of responding donors.

Epitope map of Cry3Aa. The epitope mapping assay was performed on a donor set of 47 community blood bank donors using pooled peptides describing the sequence of *Bt* Cry3Aa (Figure 3A). As seen in the Cry1Ab data set,

many peptides were associated with a 0% donor response. We calculated the background response and determined epitopes using data only from the peptides that returned responses. The background rate for the peptide set was determined to be 3.3%. This background response is consistent with our overall average, and similarly suggests that this protein is not encountered immunologically by our donor population. No peptides reached the 3-fold above background cutoff value. Two peptides returned results more than 2-fold the background, at amino acid positions 118–132 (8.5%) and 139–153 (8.5%).

Figure 3B shows the distribution of responses to the Cry3Aa peptide set. No responses were significant.

HLA associations were not determined because of the low number of responding donors.

Discussion

We tested a known food allergen, Brazil nut 2S storage protein Ber e 1, and two potential food allergens, Cry1Ab and Cry3Aa, in our epitope mapping assay. We found that the Brazil nut protein contained one major epitope in the light-chain and at least three more prominent response regions in the

heavy-chain polypeptide sequences comprising a total of 127 amino acids. As a comparison, the Cry proteins contained one major epitope in 610 amino acids (Cry1Ab) and no major epitopes in 644 amino acids (Cry3Aa). These data are consistent with the published information regarding the relative immunogenicity of these three proteins.

The human response data compiled for the Brazil nut protein peptides had a higher background rate than either of the two *Bt* Cry proteins. The background rate for Ber e 1 was higher than observed for most other proteins tested by us. This observation is consistent with protein exposure in our pool of community donors. In support of this conclusion, the background response rates for peptide sets derived from human papilloma virus proteins are also high. The background rate declines with the prevalence of the human papilloma virus strain tested (data not shown). This higher rate of background can be explained by the presence of memory responses to Brazil nuts and the human papilloma virus proteins. The epitope mapping assay uses total CD4+ T cells from our donors that have not been selected for naive versus memory phenotype. To test this hypothesis, we are retesting the Brazil nut peptide set comparing unselected and CD45RA+ CD4+ T cells (naive CD4+) from the same individuals to determine whether the presence of preactivated and/or memory CD4+ T cells has an effect on the background rate and the localization and dominance of T-cell epitopes.

The presentation of specific peptides to the immune system is controlled by the ability of processed peptides to bind to the expressed HLA class II protein. Often, this binding is promiscuous in that a given peptide can bind to many different HLA class II molecules. To compile information that may be useful for predicting the development of allergies, we determined whether there were any statistically significant associations between the presence of particular HLA molecules and responses to the epitope peptides. HLA associations have been made using this assay on other proteins—for example, *Bacillus lentus* subtilisin (Stickler et al. 2000; data not shown). We show here both positive and negative associations between HLA-DR and -DQ expression and epitope regions in the Ber e 1 protein. It would be of interest to determine if donors carrying these alleles are enriched in the Brazil nut allergic population. However, confirmation of these results with exposed donors is unlikely because the number of verified sensitized donors is small.

Our assay is based on CD4+ T-cell activation by autologous dendritic cells and 15-mer peptides derived from the amino acid sequence of the protein of interest. We set an arbitrary value of 3-fold more than the
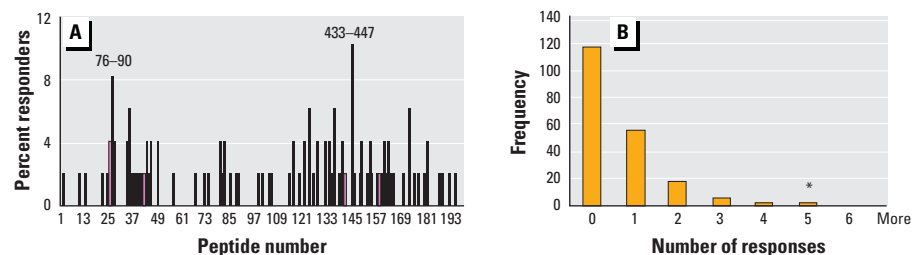

Figure 2. Mapping and designation of epitopes in *Bt* Cry1Ab. (*A*) We tested 48 community donors for reactivity with 200 15-mer peptides derived from the sequence of Cry1Ab. The peptides overlapped by 12 amino acids. Individual donor responses that reached an SI of > 2.95 were tabulated as positive. The percentage of the 48-donor pool that responded to each peptide is on the y-axis. The linear peptide designation is indicated on the x-axis. The location of the prominent peptide regions in the linear sequence of the protein is indicated. (*B*) Distribution of responses to peptides. The number of donor responses (x-axis) is plotted versus the frequency (y-axis). One peptide in the Cry1Ab data set accumulated five responses (peptide 145).
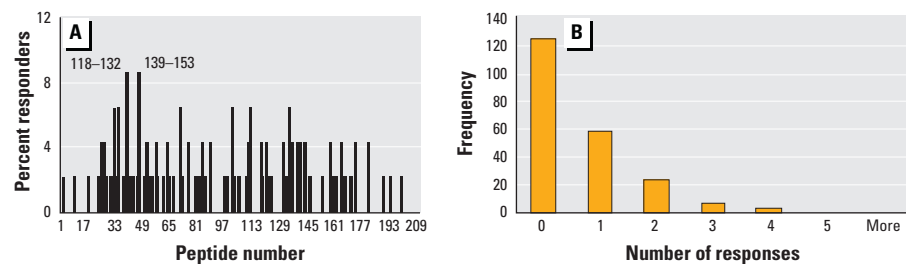*Statistically significant response.

Figure 3. Mapping and designation of epitopes in *Bt* Cry3Aa. (*A*) We tested 47 community donors for reactivity with 211 15-mer peptides derived from the sequence of Cry3Aa. The peptides overlapped by 12 amino acids. Individual donor responses that reached an SI of > 2.95 were tabulated as positive. The percentage of the 47-donor pool that responded to each peptide is on the y-axis. The linear peptide designation is indicated on the x-axis. The location of the prominent peptide regions in the linear sequence of the protein is indicated. (*B*) Distribution of responses to peptides. The number of donor responses (x-axis) is plotted versus the frequency (y-axis). No response was significant in this data set.

background for a proliferative response to a peptide to be considered positive. Data are compiled for a number of individuals, and compiled data are used to pinpoint T-cell epitopes that cause activation of T cells in the largest percentage of the population. An immunodominant ("major") epitope is defined by a percentage of the donor pool greater than or equal to 3-fold the overall background response responding to the particular sequence. This analysis compares favorably with analysis of data using background plus three standard deviations as a cutoff value. However, 3-fold greater than background indicates more epitopes than the three-standard-deviations test. Therefore, we chose to label more regions as immunodominant epitopes rather than fewer.

During the course of developing this assay we tested a large number of industrial enzymes. We verified our results using CD4^{+} T-cell samples from individuals known to have been exposed to the proteins by the presence of a positive skin-prick test. We verified our results for two industrial enzymes by testing both community donors and sensitized individuals. We are currently validating our assay against published epitopes for a third protein (not shown). We tested proteins associated with the establishment of tolerance in human donors (Tindle 2002). However, these proteins are not an appropriate control for exogenously encountered proteins, such as allergens. Regarding food allergens, we have not tested a nonimmunogenic protein because we are unaware of a protein that would meet the criteria as being verified as "nonallergenic" in all contexts. In fact, the Cry3Aa protein may be an appropriate negative control as we found no CD4^{+} T-cell epitopes in the protein sequence, and the protein is not, to our knowledge, associated with allergy.

Note that this is a technique that will delineate promiscuously binding immunodominant CD4^{+} T-cell epitopes within the donor population. Individual responses to peptides may be different from the designated common epitope regions. Therefore, modifications to proteins made based on these data will address epitopes regions for most, but not all, of the individuals tested. Modifying proteins based on our method of determining epitopes will result in the creation of hypoimmunogenic, rather than nonimmunogenic, protein variants. It is critical to appreciate this point.

In summary, we have developed an *in vitro* model to predict T-cell epitopes recognized by human cells. This assay may prove useful in the selection of potential transgenic products under consideration for the creation of genetically modified plants and foodstuffs. This assay could be extremely valuable when used in conjunction with the proposed assessments for allergenic potential of engineered food crops (Taylor and Hefle 2001).

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