A method to screen T lymphocyte epitopes after oral immunisation of humans: application to cholera toxin B subunit

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The response to oral immunisation of humans with classical biotype cholera toxin B subunit was studied to identify immunodominant T lymphocyte determinants. The in vitro proliferative response to pools of 12-mer peptides and larger peptides used individually was analysed by a novel statistical approach, and identified an immunodominant region in residues 70-79 in immunised subjects, when either pools or individual peptides were employed. In contrast, a patient infected with El Tor biotype had a dominant response to residues 40-60. The statistical software employed in this study may enable efficient screening of antigens for immunodominant T lymphocyte determinants when blood precursor frequencies are low following immunisation, and may therefore be of special relevance to mucosal vaccines.

Keywords: T lymphocyte; epitope; peptide; cholera; vaccine

Subunit vaccines should possess B and T lymphocyte epitopes, but to identify the latter, primed cells must be isolated from accessible tissues (usually blood) for in vitro proliferation studies. We found relatively high T lymphocyte precursor frequencies in blood only briefly after oral immunisation of humans with whole cholera toxin B subunit (CTB), presumably due to selective homing. We report here a statistical method which enables the rapid analysis of in vitro T lymphocyte proliferative responses to a wide range of antigenic specifities after oral immunisation with CTB, using unfractionated peripheral blood mononuclear cells (PBMCs) and pools of peptides.

MATERIAL AND METHODS

PBMCs were obtained from seven volunteers immunised orally two or more times with whole cell/CTB vaccine (classical biotype strain Inaba 569B) as reported previously. HLA DR type was determined by standardised NIH cytotoxicity assay. PBMCs were also obtained from a patient convalescing after clinical cholera (El Tor biotype strain Inaba) contracted in India.

12-mer oligopeptides with an overlap of 11 were synthesised on pins (Chiron Mimotopes, Australia) cleaved into 0.05 M HEPES buffer pH 7.8, and pooled in groups representing residues 1-21, 11-30, 20-40, 30-50, 40-60, 50-70, 60-80, 70-90 and 80-103 of CTB. Five large peptides containing residues 1-30, 20-49, 40-69, 60-90 and 80-103 were independently synthesised by standard techniques.

PBMCs (2 x 10^6) separated from fresh heparinised blood were cultured in each well of a 96 U-bottom well plate (Nunc, UK) with 200 µl of RPMI 1640 with 10% autologous serum (heated at 56°C for 30 min to remove protease activity) and 20 µg/ml gentamicin. For each subject 408 wells were set-up in one experiment as follows: replicates of 24 wells for each pool or peptide alone (0.4 µg per well (2 µg ml^-1) of each pooled peptide, or 2 µg (10 µg ml^-1) for each large peptide alone); 48 negative wells with medium and cells alone; two positive controls each of 12 replicate wells containing cells and 10 µg of CTB (Sigma Chemical, UK) or 2 µg of Concanavalin A. After 5 days at 37°C/15% CO_2, wells were pulsed with ^3H-thymidine, harvested onto glass-fibre and proliferation determined by direct beta counting (‘Matrix 96’, Packard, UK).

The immunodominant epitopes of responding T lymphocytes were identified using software (‘Alloc’, Chiron Mimotopes, Australia) in accordance with
Figure 1  (a) Precursor frequency of T lymphocytes proliferating to pools of peptides (grey bars, left-hand vertical axis) or single large peptides (black bars, right-hand vertical axis). The P.F. (expressed as number of responding cells/10⁵ PBMCs) is shown for subject infected with El Tor cholera (ET), and seven subjects immunised with classical CTB (A–G), together with the HLA-DR type. Panels have been ordered with regard to HLA-DR and magnitude of response to large peptides. (b) Relationship between residues of CTB in pools of short peptides (grey bars) and single large peptides (black bars). Residues at extreme ends of pool present in lower concentrations than centrally located residues. Predicted T lymphocyte epitopes indicated by hatched bars.

manufacturer's protocols. The statistical basis has been discussed previously. Briefly, proliferation of all wells (excluding positive controls) is used to identify a cut-off between responding and non-responding wells with not more than 0.1% of 'true negatives' above. This value is used to determine the negative/positive ratio of replicate wells for each peptide or pool and hence the Poisson mean. The Poisson mean can be used to calculate a precursor frequency at the cell number used. However, as only one cell
concentration is used, optimum cell concentration and single hit kinetics should ideally be identified in advance, as determined previously for the assay conditions used here using standard limiting dilution techniques. Data generated by the additional software option employing ‘Monte Carlo’ statistics which gave identical results is not shown.

RESULTS AND DISCUSSION

We have previously reported in vitro stimulation indices of up to 83 in response to whole CTB at the peak of T lymphocyte trafficking after oral immunisation with CTB. However, the stimulation indices fell rapidly to a plateau of around 4–10, with a precursor frequency of around 1:50 000 or less, probably due to selective homing to mucosal sites. This level of response makes it difficult to identify proliferation of T lymphocytes to individual peptides above background levels using unfractionated PBMCs, and the low precursor frequency complicates T lymphocyte cloning. To determine precursor frequencies by traditional limiting dilution analysis at several cell concentrations requires large amounts of blood. In this study the ‘Alloc’ software identified significant proliferative responses using unfractionated PBMCs with excellent correlation between pooled peptides and corresponding large peptides.

All subjects immunised orally with classical Inaba CTB had dominant responses to peptide 13 (residues 60–90), and less frequent responses to peptides 14 (80–103), and 12 (40–69), suggesting an immunodominant T lymphocyte epitope in residues 70–79 (Figure 1a). There was an excellent correlation with responses to pools, as all subjects except D responded well to pool 7 or 8 which contain residues 70–79. The program ‘T sites’ (MedImmune Inc., MA, USA) predicted two T lymphocyte epitopes residues 61–70 and 75–82, which correlate well with observed proliferation. Subject D responded mainly to pool 9 (80–103) which correlated with responses to peptides 13 and 14. Subject C also responded to these peptides, suggesting a secondary epitope in residues 80–103. A third, less dominant epitope (seen best in subject E) was associated with pool 3 (20–40) or 4 (30–50) and peptides 11 (20–49) and 12 (40–69). The ‘Alloc’ software gave excellent correlation with responses to pools and equivalent large peptides, and expansion of responding pools to identify individual 12-mer epitopes is in progress. There was no correlation with HLA-DR type, but larger numbers of patients are probably required.

In contrast, the patient infected with El Tor Inaba responded mainly to peptide 12 (40–69) and pool 5 (40–60), suggesting El Tor cholera induces a response to a different epitope, supported by poor responses to these peptides in immunised subjects. This may have arisen from differences in amino acid sequence of classical and El Tor biotypes which have been implicated in the disappointing protection induced against El Tor biotype by this vaccine, and the variable protection afforded by infection with one biotype against the other. However, the HLA type of this patient was not determined, and MHC restriction cannot be excluded as a reason for the different response. We are investigating responses to other patients infected with El Tor biotype, and these data may be important for vaccines against cholera toxin of both biotypes. This subject also responded to the minor epitopes in pools 3 and 9—peptides 11 and 13/14.

In conclusion, the ‘Alloc’ software was able to reproducibly identify an immunodominant epitope following oral immunisation with CTB within residues 70–79, which correlated well with an epitope identified by predictive algorithms (75–82). In contrast, infection with biotype El Tor induced an immunodominant epitope in residues 40–60. ‘Alloc’ may have wide application in human vaccine development to rapidly identify immunodominant regions of interest for subsequent study by T lymphocyte cloning and limiting dilution analysis, particularly in situations where precursor frequencies in blood are low such as after oral immunisation. However, for estimation of exact precursor frequencies limiting dilution analysis at different lymphocyte concentrations may still be required.

REFERENCES


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T lymphocyte epitopes of cholera toxin B subunit: L.R.R. Castello-Branco et al.