Characterization of T cell epitopes in measles virus nucleoprotein

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T cell epitopes of the measles virus (MV) nucleoprotein were studied by synthesizing overlapping 20 aa peptides over the known sequence of the protein and analysing the proliferation responses of a panel of MV-specific T cell lines and clones against these peptides. T cell lines were established from eleven healthy controls and seven multiple sclerosis patients, all with a history of past MV infection. The epitopes recognized by these lines were concentrated in a few regions of the polypeptide chain. Overlapping peptides containing aa 321–340 and 331–350 were most often recognized. Other epitopes were detected close to the amino-terminal end of the polypeptide chain as each of the peptides 1–20, 21–40, 31–50 and 51–70 contained stimulating moieties. Some responses were also detected towards peptides 151–200 and 221–250, but the carboxy-terminal end of the polypeptide was not recognized by any of the tested T cell lines. The amino acid sequences of the peptides that stimulated the T cell clones and lines, as a rule, contained binding motifs described for HLA-DR alleles found in T cell donors. The regions of protein sequence which did not reveal any T cell epitopes were, instead, relatively free of binding motifs. The results suggest that only a few epitopes of the MV nucleoprotein are important in establishing T cell immunity.

Introduction

Measles virus (MV) is an ubiquitous pathogen which is now rare in developed countries due to efficient vaccination programs but is still common in several developing countries. MV infection continues to be one of the major causes of childhood morbidity and mortality. MV causes short immune suppression which contributes to the severe complications associated with the disease, especially in undernourished children (Garenne & Aaby, 1990).

In most developed countries, children are vaccinated after 14 months of age with attenuated live vaccine. This gives a mild clinical reaction in 10–30% of immunized individuals. There are also a few contra-indications to the use of live vaccine, for instance pregnancy, known anaphylactic reactions to egg, neomycin or severely depressed cell-mediated immunity. The use of labile live vaccine in developing countries poses special problems in terms of storage and transport. A further problem in developing countries is natural infection at a very early age when the immune response is poorly induced by routinely used attenuated strains (Norrby & Oxman, 1990). Trials with high potency vaccines have indicated that the stronger immune suppressive effect of these strains may be a complicating factor (Weiss, 1992). The use of standard formalin-inactivated vaccines, on the other hand, leads to an imbalance between the components of the immune system as demonstrated by several serious cases of atypical measles (Fulginiti et al., 1967). Thus, the need to develop a safe, subcomponent or synthetic vaccine still exists and for this purpose detailed knowledge of MV structures recognized by immune cells is required (Kantz & Gellin, 1994).

Cell-mediated immunity has a critical role in the control of MV infection. In general, patients with hypogamma-globulinaemia recover uneventfully, whereas individuals with severe congenital or acquired deficiencies in cellular immune responses or combined deficiencies in cellular and humoral responses develop a progressive disease (Griffin & Bellini, 1996). The present study aimed to characterize T helper cell epitopes in the MV nucleoprotein (N protein), which is known
Fig. 1. Responses to 20 aa overlapping peptides from MV N protein by T cell lines from three healthy donors (donors A, B and C) and from one MS patient (donor D). The epitopes were mapped by using nine pools of six 20 aa peptides in the first phase (a–d) and dissecting the responses by including single peptides of stimulating pools in the second test (e–h). MV, culture with measles virus; med, culture without antigen.
to be a main target of MV-specific T cell response (Ilonen et al., 1990). This was accomplished using MV-specific T cell lines and clones and a panel of overlapping synthetic peptides prepared according to the known amino acid sequence of the N protein.

Methods

Study subjects. Healthy laboratory staff members with a history of a past MV infection as well as patients with confirmed multiple sclerosis (MS) donated blood for the study.

MV and synthetic peptides. To prepare MV antigen, a wild-type isolate of MV (Halonen strain; Rota et al., 1994) was grown in Vero cells and inactivated with β-propiolactone as described previously (Ilonen, 1979). Fifty-two synthetic peptides with a length of 20 aa and 10 aa overlaps covered the whole MV nucleocapsid sequence of the Edmonston vaccine strain (Rozenblatt et al., 1985; Taylor et al., 1991). The peptides were synthesized using t-Boc amino acids (Bachem) and p-methylbenzhydrylamino acid (Fluka) according to the multiple solid-phase synthesis method (Houghten, 1985). Removal of the protecting groups from the formyl-tryptophan and methionine sulfoxide residues was achieved by cleavage with 25% hydrogen fluoride (Tam et al., 1983). The peptides were then cleaved from the resin with liquid hydrogen fluoride using a multi-vessel apparatus (Houghten et al., 1986).

T cell lines. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of MV seropositive subjects by Ficoll–Paque gradient centrifugation. PBMC (2 x 10⁶ per ml) were incubated with the MV antigen (10 µg/ml) in RPMI 1640 medium supplemented with 7.5% human AB serum, gentamicin sulfate (10 µg/ml), HEPES buffer solution (1 M, 20 µl/ml) and 3% glutamine (10 µl/ml). After 7 days, fresh medium supplemented with IL-2 (20 U/ml) was added every second or third day. After a total of 17 days, the T cells were restimulated with both MV antigen and irradiated (30 Gy) autologous antigen-presenting cells (APC; 2 x 10⁶ PBMC/ml). T cell lines were restimulated at 10 day intervals, IL-2 was added 2 days after antigen stimulation and at 2–3 day intervals thereafter. T cell lines had more than two cycles of stimulation with virus antigen. The majority of the T cells (mean 82.9% from nine lines) were CD4⁺ when the phenotype was analysed by flow cytometry (Facsscan; Becton Dickinson) using FITC- or phycoerythrin-conjugated MAbs (Coulter) to the CD4 and CD8 T cell surface markers.

T cell clones. Six MV-specific clones were established from three donors. MV-specific T cell lines were cloned 5–10 days after the last antigen stimulation. Antigen-specific T cell lines were first incubated in Terasaki plates in 20 µl 10% AB medium supplemented with IL-2 and containing 1 x 10⁴ irradiated feeder cells and 10 µg/ml MV antigen. After 10 days, the growing cells were transferred to flat-bottomed 96-well microtiter plates and IL-2, APC and MV antigen were added. Thereafter, clones were stimulated and IL-2 was added as described above for the antigen-specific T cell lines. HLA-restriction (DR1 or DR2) and specificity of the five clones for the N protein have been described previously (Ilonen et al., 1990). All the randomly selected MV-specific T cell clones were positive for CD3 and CD4 but negative for CD8 markers (Ilonen et al., 1990).

Lymphocyte proliferation assay. A total of 1 x 10⁴ T cells was incubated in triplicate wells with 2 x 10⁴ APC (PBMC) and different antigens in 200 µl volumes in 96-well round-bottomed microtitre plates for 2 days. MV antigen or N protein peptides were used at a final concentration of 10 µg/ml. Tritiated thymidine (0.5 Ci/ml) was added 18 h before harvesting the cultures. Incorporated radioactivity was measured using a MicroBeta scintillation counter (Wallac Oy). A stimulation index was calculated by dividing the median of stimulated triplicate culture wells by the median of the control responses. A response was considered positive when the stimulation index was higher than 3.

HLA restriction studies. To define the restriction element of each MV-specific T cell clone, cells were incubated with MV antigen and with the APC which were selected to share different DR alleles with the T cell line donor. Cells without any common DR alleles and autologous cells served as negative and positive controls, respectively.

HLA typing. HLA typing of blood donors was done using panels of
commercial antisera (Biotest) in a standard microlymphocytotoxicity test. B cells for HLA class II typing were enriched using immunomagnetic beads (Dynal).

**Results**

**Identification of T cell epitopes on MV N protein**

MV-specific T cell lines recognizing N protein were established from eleven healthy controls and seven MS patients. The epitopes detected by these lines were mapped using nine pools of six 20 aa peptides in the first phase and dissecting the responses by including single peptides from the stimulating pools to the second test. The results revealed large inter-individual variation among the 18 T cell lines but no variability was observed between experiments on the same lines. Fig. 1 demonstrates the mapping of epitopes recognized by four individual T cell lines according to this scheme. New lines were established from two donors a few years after the first. The peptides recognized by these new lines were also stimulated using the original lines, but all epitopes of the first experiment were not detected by the new lines (data not shown).

The responses to N protein-derived peptides were concentrated in a few regions of the polypeptide chain (Fig. 2). Peptides 321–340 and 331–350 evoked responses most often; peptide 321–340 gave a response in five healthy controls but not in any of the seven MS patients. The difference between the MS patients and controls did, however, not reach statistical significance ($P > 0.1$, Fisher’s exact test). Other epitopes were detected close to the amino-terminal end of the polypeptide chain, each of the peptides 1–20, 21–40, 31–50 and 51–70 containing stimulating sequences. Responses were also detected to peptides 151–170, 161–180, 171–190, 181–200, 221–240 and 231–250, but the carboxy-terminal end of the polypeptide was not recognised by any of the T cell lines.

Six N-specific T cell clones responded to the same peptide epitopes that were detected by the T cell lines. Two HLA-DR2-restricted clones responded to the overlapping peptides 21–40 and 31–50. One DR2-restricted clone detected an epitope in peptide 161–180. The clone restricted by HLA-DR12(5) recognized peptides 221–240 and 231–250 (Fig. 3) and two DR1-restricted clones recognized peptide 51–70.

**MV N protein epitopes and HLA-DR motifs**

The overlapping region of peptides 21–40 and 31–50 recognized by two DR2-restricted clones from two different donors has an amino acid sequence **IKHIIIV** (actual motif shown in bold) which exactly fits the DRB1*1501 (DR2) motif with the first isoleucine as a hydrophobic amino acid in the P1 position (Rammensee et al., 1995). There is also another possible DR2 epitope in peptide 21–40, **IRGIKHL**. One HLA-DR2-restricted clone recognized peptide 161–180 where a DRB1*1501 motif **LAQIWL** can also be found. The epitope shared by the overlapping peptides 221–240 and 231–250 and recognized by the HLA-DR12(5)-restricted clone has the DRB1*1201 motif **FMVALLD**. Peptide 51–70, recognized by two HLA-DR1-restricted clones from different donors, does not reveal an exact DRB1*0101 motif but the sequence **LVRLIGNPD** has leucine in positions 1 and 4 and glycine in position 6, which match the motif. This peptide also stimulated four T cell lines. Two of these lines were HLA-DR1-positive.
Table 1. Peptides recognized by MV-specific human T helper cell lines

Fifty-two overlapping 20 aa peptides were synthesized according to the sequence of the MV N protein.

<table>
<thead>
<tr>
<th>Peptides recognized</th>
<th>HLA-DR type of blood donor</th>
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<tbody>
<tr>
<td>aa 1–20</td>
<td>DR1,2; DR2,6</td>
</tr>
<tr>
<td>aa 21–40</td>
<td>DR2,5; DR1; DR4,12(5); DR4,8</td>
</tr>
<tr>
<td>aa 51–70</td>
<td>DR4,8; DR4,6; DR1; DR1,2</td>
</tr>
<tr>
<td>aa 151–170</td>
<td>DR1,2</td>
</tr>
<tr>
<td>aa 161–180</td>
<td>DR4,6; DR1,2</td>
</tr>
<tr>
<td>aa 171–190</td>
<td>DR4,6; DR1; DR8,11(5)</td>
</tr>
<tr>
<td>aa 181–200</td>
<td>DR4,6</td>
</tr>
<tr>
<td>aa 221–240</td>
<td>DR2,8</td>
</tr>
<tr>
<td>aa 321–340</td>
<td>DR7,13(6); DR1,2; DR1,7; DR1,11(5); DR12(5)</td>
</tr>
<tr>
<td>aa 331–350</td>
<td>DR1,11(5); DR1; DR1,2; DR1,7; DR7,13(6); DR2,7; DR4,7; DR9; DR12(5); DR2,8</td>
</tr>
</tbody>
</table>

and the two others had HLA-DR4 in common (Table 1). There are also elements of DR4 motifs in this peptide: LDRLVRGLG and VRLGNPD with appropriate amino acids for the P1, P4 and P6 positions of the motif.

All of the four lines from DR7-positive donors (two controls and two MS patients) showed a response to peptide 331–350 in which VELENS fits the HLA-DR7 motif i + 5 (Chicz et al., 1993). Peptide 321–340 has another DR7 motif, YPLLWS. Samples from three of the five donors that recognized peptide 321–340 have HLA-DR1 and this peptide has the DRB1*0101 motif (YPLLWSYAM) described by Hammer et al. (1993). Samples from four of the eleven donors that were HLA-DR1-positive recognized peptide 331–350 with the HLA-DR1 motif (WSYAMGVGV) described by Rammensee et al. (1995).

Altogether 56% of the peptides stimulating the T cell lines exactly fit motifs listed by Rammensee et al. (1995). The addition of the DR7 and DR8 motifs not included in the former list but described by Chicz et al. (1993) increases the percentage to 67%. Fig. 4 summarizes the HLA binding motifs used in the N protein sequence, revealing that the regions which did not contain T cell epitopes were also relatively free of class II binding motifs.

Discussion

T cell epitopes which could be identified in the present study using a panel of MV-specific T cell lines and clones were concentrated to a few sites within the MV N protein and the lack of epitopes at the carboxy-terminal end of the molecule was especially conspicuous. This is in accordance with the general principle of immunodominance which states that the T cell response to a foreign protein is directed to a limited number of determinants (Berzofsky, 1988). Only a few immunogenic epitopes have, as a rule, been found in various microbial proteins (Banos et al., 1997; Burkhart et al., 1994; Callebaut et al., 1993; Marttila et al., 1996; Obeid et al., 1993; Vordermeier et al., 1992).

Our study was designed to map epitopes recognized by CD4+ T helper cells. Generally, this T cell subpopulation is activated using exogenously added antigen (Yewdell & Bennink, 1990), as also demonstrated by the CD4 positivity of MV-specific T cell clones prepared earlier with the same protocol (Ilonen et al., 1990). The earlier work by Nanan et al. (1995) identified three cytotoxic T cell epitopes in the N protein. These were contained in sequences 210–218, 226–234 and 340–348. Two of these are close to the T helper epitopes found in this study; the most commonly recognized epitope...
was within peptide 331–350, and peptide 221–240 also stimulated one T cell line. The flanking localization of CD4 and CD8 epitopes has been found in rubella virus E1 protein (Nepom et al., 1997). This type of phenomenon might facilitate the eventual development of synthetic vaccines containing a few selected peptide stretches.

There is a recent study describing T helper cell epitopes in MV N protein with results that differ from those reported here. Hickman et al. (1997) used synthetic peptides to stimulate directly lymphocytes from the peripheral blood of subjects recently vaccinated or recovered from natural MV infection. They found that epitopes were evenly distributed over the whole amino acid sequence with a particular concentration at the carboxy-terminal end. In our study, this area was devoid of epitopes. The number of epitopes recognized by single subjects was also large in some individuals. The method using synthetic peptides to directly stimulate peripheral blood cell lymphocytes is complicated by the fact that a very wide cross-reactivity at the T cell level between various microbial and autoantigens has been described (Wucherpfennig & Strominger, 1995). Peptides eventually eliciting responses are not necessarily processed from a native protein (Deng et al., 1993). Our method of creating T cell lines with whole viral antigen ensures the recognition of true viral structures by the tested T cells. The correlation between the T cell epitopes found and the binding motifs of the common HLA-DR alleles covered by the panel was remarkably good. Stimulating peptides were found to fulfill the criteria of binding motifs, although only some of the peptides with these motifs were actually able to stimulate T cells. This is in accordance with the recent results of Gelder et al. (1998), which revealed that only a few influenza virus A haemagglutinin peptides that bound to MHC molecules were real T cell epitopes.

The subjects in our study had past experiences of natural MV infection and one might speculate that the recent infection or vaccination analysed by Hickman et al. (1997) might be associated with a wider panel of recognition than long-term immunological memory. Our study was designed to find immunodominant epitopes that would be recognized in the context of common class II HLA alleles. Primarily, only one line was established from each subject and it is possible that some clones became dominant during the in vitro culture period. We also, however, have experience from mapping other virus epitopes in rubella virus (Marttila et al., 1996) and coxsackie B4 virus (J. Marttila and others, unpublished results), which demonstrate the stability of recognized major epitopes. The two lines which were raised from the original donors several years after the first experiment still recognized previously found epitopes. The stability of the T cell approach in our hands is good compared to experiments we have made stimulating PBMCs with synthetic peptides.

A potential problem in our study is created by the differences between MV strains. There is variability in the N sequence between different MV isolates. This variability is especially concentrated in the carboxy-terminal end which was particularly devoid of epitopes in our study. There was also a notable lack of possible HLA-DR motifs in the carboxy-terminal end of the N sequence in the analysis which included the most common DR alleles. We do not know the sequences of the MV strains which induced the immunity in the study subjects, but the Finnish wild-type isolate (Halonen strain) we used as the antigen is the best available representative of the last circulating strains. The Finnish wild-type isolate and the Edmonston wild strain have an identical gene for the N protein (Rota et al., 1994) and differed from the vaccine strain only in 7 aa. This makes it unlikely that differences in the sequence between wild-type viruses and the Edmonston vaccine strain used for peptide synthesis might explain the lack of responses.

In conclusion, we have demonstrated that only a few immunodominant regions of the MV N protein sequence are important in long-term T helper cell memory. This information is important in the development of new vaccines and in elucidating the immunopathological complications associated with MV infection, although the results of the present study are still preliminary due to the relatively small number of subjects in the study group.

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References


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