Identification of T-Helper Epitopes in the VP1 Capsid Protein of Poliovirus

M. KUTUBUDDIN,1 J. SIMONS,2 AND M. CHOW1

Departments of Biology1 and Applied Biological Sciences,2 Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received 16 September 1991/Accepted 20 February 1992

Poliovirus-specific T lymphocytes were isolated from virus-immunized mice of different H-2 haplotypes. Immunological characterization of this population indicates that the effector population involved in the observed poliovirus-specific proliferative response was that of CD4-positive T-helper cells. Proliferative responses also were induced within these T-lymphocyte populations upon stimulation with either purified VP1 capsid protein or VP1 synthetic peptides. By using these synthetic peptides, several T-helper epitopes were identified. Generally, proliferative responses were observed in three regions of VP1. Two regions spanning VP1 residues 86 to 120 and 201 to 241 were recognized by T lymphocytes from BALB/c (H-2b), C57BL/6 (H-2d), and C3H/HeJ (H-2k) backgrounds. Analyses using synthetic peptides of nonoverlapping sequences indicated that the region spanning residues 201 to 241 may contain several T epitopes and may account for the strong proliferative response observed. In addition, for two of the three haplotypes examined, T epitopes were observed within residues 7 to 24 of VP1. Additional epitopes which appeared to be restricted to specific H-2 backgrounds were identified. T epitopes within VP1 that are common between different strains of mice appeared to lie within previously identified neutralizing antigenic sites in poliovirus.

The immune response to many viral infections is characterized by induction of virus-specific humoral and/or cell-mediated immunity. For poliovirus, the humoral immune response is considered to form the basis of protective immunity against viral infections in humans. Extensive characterization of this humoral response in mice has identified regions of the viral capsid recognized by neutralizing antibodies (4-6, 8, 15, 22-24, 26, 33-35). A major component in the development of an effective humoral response is the immunological help provided by antigen-specific T-helper (Th) cells. CD4+ helper T lymphocytes recognize foreign antigen fragments (Th epitopes) in the context of self major histocompatibility complex class II molecules. Such Th epitopes are distinct from B epitopes or antibody-binding sites. It is therefore important to define the Th epitopes present on the viral capsid proteins. Although little is known about the Th response upon host exposure to poliovirus, the absence of an antibody response in athymic mice demonstrates its contribution to the development of an effective poliovirus-specific humoral response (14). Recently, a Th epitope was identified in capsid protein VP1 of poliovirus serotype 1, Mahoney strain (PVM) (17). The present study was undertaken to analyze more extensively the murine T-cell response to poliovirus. Several Th epitopes were identified in the VP1 capsid protein for different inbred strains of mice. Interestingly, most of the Th epitopes of VP1 identified were found to lie close to previously identified neutralizing antibody-binding sites.

MATERIALS AND METHODS

Cells and media. HeLa cells in suspension culture were maintained in Joklik’s modified minimal essential medium supplemented with 5% horse serum (GIBCO). Virus stocks were stored and diluted in phosphate-buffered saline. T-lymphocyte cultures were maintained in RPMI 1640 supplemented with 2-mercaptoethanol (5 × 10⁻⁵ M) and 2% normal mouse serum.

Virus propagation and purification. PVM was grown by infecting suspension HeLa cells with a virus stock grown at low multiplicity from a plaque derived by transfection of the infectious cDNA (25). Virus was purified by banding on cesium chloride gradients, and virus concentration was estimated by determining the optical density at 260 nm and using an extinction coefficient of 7.7 optical density units per mg per ml (30).

Preparation of viral antigens. Capsid proteins VP1, VP2, and VP3 were isolated by electrophoresis from sodium dodecyl sulfate-polyacrylamide gels (5). The concentrations of the eluted proteins were estimated in polyacrylamide gels by the staining intensities of the bands relative to those of capsid protein standards of known concentrations. Synthetic peptides of VP1 sequences were synthesized with solid-phase methods by using a Beckman 990b peptide synthesizer. Most of these peptides were used previously to identify neutralizing epitopes in VP1 (6). Peptides were dissolved in the culture media and used at a concentration of 10 μg/ml.

Immunization and T-lymphocyte isolation. BALB/c (H-2d), C57BL/6 (H-2b), and C3H/HeJ (H-2k) female mice (4 to 6 weeks old; Jackson Laboratories) were immunized with 2 μg of poliovirus in complete Freund’s adjuvant in a volume of 100 μl subcutaneously at the base of the tail. The mice were killed 10 to 14 days following immunization, and the inguinal lymphode nodes (LN) were removed aseptically. Single-cell LN suspensions were prepared by squeezing the LN in a tube with a loosely fitting Teflon rod (2, 18). Cells were cultured at a density of 2 × 10⁶ per well in 96-well round-bottom culture plates (Costar) in the presence of stimulating antigen.

In vitro antigen-specific T-cell proliferation assays. Bulk T-cell cultures were incubated in the presence of antigen for 3 days at 37°C in 7.5% CO₂. Purified poliovirus virions or synthetic peptides were used as the stimulating antigen at
different concentrations. To assay for T-cell proliferation in these antigen-stimulated cultures, triplicate cultures were labeled for 16 h with 1 μCi of [3H]thymidine (2 Ci/mmol; final concentration 1 μCi/0.2 ml; NEN). Cells were harvested on glass fiber filters by using a cell harvester (Skatron), and the incorporated [3H] radioactivity was quantitated by using a toluene-based scintillation fluid. Results are expressed as the mean of triplicate cultures ± the standard deviation. Stimulation indices (SI) were determined as the ratio of [3H]thymidine incorporated in antigen-stimulated versus unstimulated control cultures. Positive responses were identified as those with SI of greater than 2.

Immunological characterization of T-lymphocyte cultures. Monoclonal antibodies (MAb) against the CD4, CD8, and Thy1.2 markers on murine T cells were used to define the T-lymphocyte population. GK1.5 is a rat hybridoma specific for the L3T4 (CD4) marker (7). MAB 3.15S is a CD8-specific rat hybridoma (31). HO13.4 is a mouse anti-Thyl.2 hybridoma (29). Serial dilutions of the hybridoma culture supernatants were added, together with poliovirus (1 μg/ml), to the T-lymphocyte cultures. T-cell proliferation was assayed 3 days later by thymidine incorporation.

Prediction of Th epitopes in VP1 capsid protein. The primary amino acid sequence of VP1 capsid protein of PVM was used for prediction of Th epitopes. Sites of hydrophobic variation consistent with formation of amphipathic segments were determined by using the AMPHI algorithm (19). Calculations used the Fauchere-Pliska hydrophlicity scale (9), a block length of 7, and an amphipathicity score threshold of 8. The occurrence of Roohard-Taylor motifs also was analyzed in the amino acid sequence of VP1 by scanning the sequence for a charged or glycine residue followed by two or three hydrophobic residues and subsequently a polar, charged, or glycine residue (27).

Nomenclature. By convention, each amino acid residue within the capsid proteins is designated by using a four-digit number with the first digit identifying the capsid protein and the last three identifying the residue number. Thus, amino acid residues 1 through 302 from VP1 are designated 1001 through 1302.

RESULTS

Antigen-specific T-cell proliferative responses to poliovirus and capsid proteins. T lymphocytes proliferate in vitro upon stimulation with antigen. Thus, to establish that poliovirus-specific T-cell populations were induced upon immunization with infectious virus, the inguinal lymph node (LN) cells of immunized BALB/c mice were isolated, cultured in the presence of different concentrations of virus, and assayed for T-cell proliferation (Fig. 1). Specific proliferative responses to poliovirus were observed in these cultures with SI of greater than 2; these responses titrated with decreasing virus concentrations. To assess the relative contribution of T epitopes from different capsid proteins, T-cell proliferative responses were determined in the presence of purified VP1, VP2, or VP3 capsid protein as the stimulating antigen. A prominent T-cell response to VP1 capsid protein was observed with SI of greater than 2. Similar responses to VP1 were observed inLN cultures isolated from poliovirus-immunized C57Bl/6 and BALB/c mice (data not shown). Small stimulatory effects were observed consistently also with purified VP2 and VP3 capsid proteins, indicating the presence of T epitopes in these proteins. However, because the SI observed with VP2 and VP3 were never above 2, further characterization was difficult. In Western blot (immunoblot) analysis, the sera from these mice appeared to contain antibodies only to VP1, suggesting that the specific immunization protocol used in these studies may preferentially elicit VP1-specific immune responses (data not shown). Thus, the low proliferative responses to VP2 and VP3 observed may be due to the early predominance of VP1 immune responses rather than to an absence of T epitopes in VP2 or VP3.

Antibody blocking of in vitro lymphocyte proliferation in response to poliovirus. The effector population involved in the proliferative response was characterized by using MAb that recognize the Thy1.2, L3T4, and CD8 surface markers. LN cells from poliovirus-immunized BALB/c mice were cultured in vitro in the presence of virus and serial dilutions of the hybridoma supernatants and subsequently assayed for proliferation responses (Fig. 2). Consistent with the hypothesis that the response is due to a population of T lymphocytes present in the culture, virus-specific proliferation was effectively blocked in the presence of the anti-Thy1.2 MAb at all dilutions. The Thy1.2 marker was found on the surface of all mature T cells, T-cell precursors, and thymocytes and not on that of B lymphocytes. T-cell proliferation was effectively inhibited by the anti-L3T4 MAb and not by the anti-CD8 MAb at the same concentrations, indicating that the effector population in the LN cells involved in the poliovirus-specific proliferative response is that of CD4-positive T cells.

VP1 sequences recognized by Th cells. The strength of the proliferative response observed upon stimulation with purified VP1 capsid protein allowed further identification of the contributing epitopes. T lymphocytes were isolated from virus-immunized BALB/c mice and cultured in vitro with synthetic peptides of VP1 sequences as the stimulating antigen (Fig. 3A). These synthetic peptides are from differ-
ent regions of the VP1 protein and represent, in aggregate, approximately 90% of the VP1 capsid sequence. In addition, a synthetic peptide from the influenza virus type A hemagglutinin (amino acid residues 201 to 223) was used as a control peptide at the same concentration (12). Consistent with the previous report (17), peptide 12a (spanning VP1 amino acid residues 1100 to 1109) induced proliferative responses with SI of greater than 2. In addition, peptides 7, 11, and 14 (spanning VP1 sequences 1202 to 1221, 1222 to 1241, and 1007 to 1024, respectively) also induced strong proliferative responses. (Because there is no apparent sequence homology between peptides 7 and 11, the positive proliferative responses observed suggest that both peptides contain T epitopes.) The responses were titratable and were not observed with naive mice (data not shown). All other peptides reproducibly failed to induce T-cell proliferation in these assays. Thus, three regions in VP1 (residues 1007 to 1024, 1100 to 1109, and 1202 to 1241) appear to be recognized by virus-specific T lymphocytes from BALB/c mice.

The VP1 synthetic peptides were also used in proliferation studies to identify the T epitopes induced in other inbred mouse strains upon exposure to poliovirus (Fig. 3B and C). Similar to the response observed in BALB/c mice, poliovirus-specific T lymphocytes from C57BL/6 mice proliferated in response to antigen stimulation with peptides 7 and 11. In contrast, no proliferation was detected with either peptide 14, 12a, or 12b, nor with peptide 3, whose sequences overlap those of peptides 12a and 12b. However, peptides 2 (spanning residues 1061 to 1080) and 15 (spanning residues 1110 to 1120) consistently stimulated proliferation in the C57BL/6 cultures. Thus, although the exact regions differ from those observed in BALB/c mice (H-2k), C57BL/6 (H-2b) T lymphocytes also recognize three regions from VP1 (spanning 1061 to 1080, 1110 to 1120, and 1202 to 1241) and the major proliferative response was also observed with peptide 7. A more diffuse response was observed in the bulk T-lymho-

cyte cultures from virus-immunized C3H/HeJ mice (H-2d). Although peptide 7 failed to induce proliferation, peptide sequences adjacent to peptide 7 (peptides 6, 11, and 8, which span residues 1182 to 1201, 1222 to 1241, and 1244 to 1264, respectively) induced detectable proliferative responses. Again, no sequence homology was present between peptides 6, 11, and 8, suggesting that this region of VP1 contains multiple T epitopes. Peptides 14 (spanning residues 1007 to
Table 1. Summary of predicted amphipathic segments in VP1 capsid protein

<table>
<thead>
<tr>
<th>Segment no.</th>
<th>Midpoint of blocka</th>
<th>Angle (°)b</th>
<th>Amphipathicity score</th>
<th>Amphipathic segment</th>
<th>Amino acid sequencec</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-18</td>
<td>80-120</td>
<td>52.0</td>
<td>1-21</td>
<td>GLGQMLMSIDNTVRETVGAA</td>
</tr>
<tr>
<td>2</td>
<td>77-80</td>
<td>80-105</td>
<td>8.9</td>
<td>74-83</td>
<td>ESBSBFPFAR</td>
</tr>
<tr>
<td>3</td>
<td>87-89</td>
<td>105-120</td>
<td>8.6</td>
<td>84-92</td>
<td>GACVITMV</td>
</tr>
<tr>
<td>4</td>
<td>94-97 (P)</td>
<td>105-120</td>
<td>9.2</td>
<td>91-100</td>
<td>TVDNPASSTTN</td>
</tr>
<tr>
<td>5</td>
<td>151-157</td>
<td>95-120</td>
<td>17.2</td>
<td>148-160</td>
<td>GHALNQYQIMYV</td>
</tr>
<tr>
<td>6</td>
<td>168-171 (P)</td>
<td>90-120</td>
<td>9.8</td>
<td>165-174</td>
<td>FPVEEKWDYYT</td>
</tr>
<tr>
<td>7</td>
<td>200-213 (K, P)</td>
<td>90-125</td>
<td>35.7</td>
<td>197-216</td>
<td>FTYGINSYSHYQFQSKVP</td>
</tr>
<tr>
<td>8</td>
<td>225-230</td>
<td>80-120</td>
<td>12.5</td>
<td>222-233</td>
<td>AALGSDLVQAGS</td>
</tr>
<tr>
<td>9</td>
<td>272-278 (P)</td>
<td>90-135</td>
<td>15.5</td>
<td>269-281</td>
<td>WCPPFPAYAVYG</td>
</tr>
</tbody>
</table>

a The amphipathic character of VP1 sequences was iteratively examined from the N-terminal glycine in blocks of seven amino acids. Amphipathic segments are denoted by the midpoint positions of the identified blocks. Thus, the predicted sites extend on each side for three residues. P or K indicates the presence of proline or lysine in the amphipathic segment.

b The angle describes the pitch of the helix that is predicted by the amphipathic segment.

c The Rothbard-Taylor motifs present within these amphipathic segments are underlined.

1024) and 1 (spanning residues 1024 to 1040) elicited significant proliferative responses, suggesting the presence of a second T-epitope-rich region in the N terminus of VP1. In addition, the SI observed with peptide 3 was significantly above the background. The peptide 3 sequence overlaps those of peptides 12a and 12b. Thus, although the C3H/HeJ response is more dispersed among the peptides examined, regions similar to those recognized in peptides examined, regions similar to those recognized in BALB/c mice also appear to contain T epitopes recognized by these mice. Interestingly, in each of the mouse strains examined, multiple Th epitopes appeared to be present in the VP1 region surrounding residues 1202 to 1241.

Prediction of Th epitopes in VP1. Theoretical approaches have been used to identify putative Th epitopes. One approach is based on observations that previously identified T epitopes were highly amphipathic in character and predicts T epitopes within a specific amino acid sequence on the basis of the propensity of amino acid sequences to form amphipathic segments (19). Examination of the VP1 sequence by using the AMPHI algorithm identified nine amphipathic segments which form seven potential T-epitope regions (Table 1). Eight of the predicted amphipathic segments were represented by the panel of synthetic peptides used in T-cell proliferation assays. Of these eight predicted segments, the synthetic peptides from six of these regions induced T-cell proliferative responses in at least one strain of mice examined; peptides from two of the predicted segments failed to induce proliferative responses (Fig. 4). However, three of the epitopes observed were not predicted by any of these algorithms. Most noticeably, peptide 12a was not predicted by the AMPHI algorithm. Thus, no strong correlation between the T epitopes identified and those predicted solely on the basis of their amphipathic character was observed.

The predictive value of the amphipathic segments is increased by the presence of Rothbard-Taylor motifs within these segments (27). These are tetramer or pentamer sequences with the general structure GXXZ or GXXXXZ, where G is a glycine or charged residue, X is any hydrophobic residue, and Z is a glycine, charged, or polar residue. Examination of the VP1 sequence identified 12 tetramer and 4 pentamer sequences as Rothbard-Taylor motifs. Only two tetramer motifs (HYFD and GAAS) and one pentamer motif (RAVAY) were found to lie in amphipathic segments, which are represented by peptides 7, 11, and 9 (Table 1). Although no T-cell responses were observed with peptide 9, it is interesting that the region represented by peptides 7 and 11 induced strong proliferative responses in all three strains of mice examined.

**DISCUSSION**

Poliovirus-specific Th lymphocytes were isolated from virus-immunized mice of different H-2 haplotypes. Strong proliferative responses were induced within these T-lymphocyte populations upon stimulation with either purified VP1 capsid protein or VP1 synthetic peptides. By using the synthetic peptides, the locations of new Th epitopes were identified. Broadly, peptides which are recognized by multiple H-2 haplotypes and appear to identify the major VP1 Th epitopes lie in three regions (Fig. 4).

One region, upon stimulation with peptides, induced strong proliferative responses in these bulk T-cell cultures. This region surrounds residues 1202 to 1241 of VP1. The relative predominance of responses observed in all three haplotypes to peptides from this VP1 region may be due to several factors. The predominant response to peptide 7
observed in BALB/c and C57BL/6 mice suggests that an immunodominant T epitope is located within this region. In addition, the proliferative responses induced by peptides 7 and 11 in BALB/c and C57BL/6 mice and by peptides 6, 11, and 8 in C3H/HeJ mice demonstrate that multiple T epitopes reside within this region. It is possible that any one peptide derived from this highly antigenic region includes more than one T-cell epitope. Thus, T lymphocytes of different epitope specificities may have contributed to the peptide response observed. Characterization of poliovirus-specific T-cell clones will help clarify these issues.

Peptides from a second region, surrounding residues 1086 to 1120, induced proliferative responses in BALB/c, C3H/HeJ, and C57BL/6 mice. A similar Th epitope, which was reported to be present only in BALB/c mice, was identified previously by using a nested set of synthetic peptides in the region from 1103 to 1115 (17). Our results are consistent with these previous findings and together indicate that this poliovirus Th epitope in BALB/c mice is located within the seven-amino-acid sequence KLFAYVKK. Consistent with this location, peptide 15 (spanning residues 110 to 120) failed to induce proliferative responses in BALB/c mice. In addition, a comparable epitope appears to lie further toward the amino terminus (within residues 1086 to 1103) in C3H/HeJ mice and further toward the carboxy terminus (within residues 1110 to 1120) in C57BL/6 mice. The previous failure to identify this epitope in C3H/HeJ mice most likely occurred because these sequences were not represented in the nested set of synthetic peptides tested (17). The failure of peptide 12b (spanning residues 1091 to 1109) to induce T-cell proliferation responses in BALB/c mice is surprising because this peptide includes the proliferation-positive 12a peptide sequence. Although the reason for this discrepancy is unclear, the nonresponsiveness was reproducible with several different peptide preparations. Thus, this effect is not likely due to a technical artifact but is specific to the 12b peptide sequence. One possibility is that peptides 12a and 12b are processed differently such that the Th epitope sequence in 12b is lost (or destroyed). Alternatively, the 12a peptide (10 residues) is comparable in size to those found endogenously in cells associated with major histocompatibility complex molecules (3). Peptide 12b, which is 19 residues long, may not bind to the major histocompatibility complex molecule with as much affinity as does 12a and thus is not presented as efficiently as the decapetide 12a.

Several additional epitopes appeared to be restricted by the haplotype of the strain examined. For each of these peptides, the proliferative responses were not consistently as strong as those seen in the major VP1 Th epitope regions (discussed above). It should be noted that the T-epitope sequences identified in this study are biased by the peptides tested. It is possible that if peptides whose sequences spanned the junctions of the peptides used in this study had been used, similar strong responses might have been observed in the other regions.

T epitopes have been predicted on the basis of their amphipathic nature or the presence of Rothbard-Taylor tetramer-pentamer motifs. The high amphipathicity and the presence of Rothbard-Taylor tetramer-pentamer motifs are thought to define parameters which are common for efficient binding of an epitope with different major histocompatibility complex molecules (28, 32). Usually, peptides containing these predicted sequences are subsequently synthesized and the presence of T epitopes is tested. The peptides used in this study spanned virtually the entire VP1 sequence and were not selected on the basis of these predictions. Thus, they provide a system to test the accuracy of these predictive methods. No strong correlation, within a specific H-2 haplotype, was detected between the Th epitopes observed and those predicted on the basis of their amphipathic nature or the presence of Rothbard-Taylor tetramer-pentamer motifs. Peptide sequences containing T epitopes which were not predicted were identified; conversely, some predicted sequences also failed to stimulate a proliferative response in these poliovirus-specific T-cell cultures.

Two of the major Th epitope regions (residues 1086 to 1120 and 1202 to 1241) were recognized by each of the three haplotypes tested. The locations of these Th epitope regions in VP1 are striking because these regions were identified previously as neutralizing antigenic sites on the virus. Neutralizing antigenic sites have been identified by using a series of viral mutants that escape neutralization by MAb isolated from virus-immunized BALB/c mice (8, 22–24, 26, 33–35). On the basis of the nomenclature of Page et al. (26), neutralizing site 2 is formed largely by the interaction of two loop structures, one from VP2 residues 160 to 172 (i.e., residues 2160 to 2172) and one from VP1 residues 1220 to 1226. The sequences in the VP1 component of neutralizing site 2 are spanned by peptides 7 and 11. Similarly, peptides 3 and 12a (residues 1086 to 1103 and 1100 to 1109, respectively) contain VP1 sequences that are part of neutralizing site 1 (which includes residues 1091 to 1104). In addition, peptides 11 and 12a (when coupled to keyhole limpet hemocyanin) induce serotype-specific poliovirus-neutralizing antibodies in immunized rats and rabbits (6). Thus, the T epitopes recognized by the three murine haplotypes appear to be coincident with previously defined neutralizing antigenic sites. In addition, although positive Th responses were observed with peptide 2 in C57BL/6 mice only, this peptide (coupled to keyhole limpet hemocyanin) also induces a neutralizing antibody response in rats and rabbits (6). Thus, a T-cell epitope appears to be located within this neutralizing antigenic site. It is unknown whether a neutralizing antigenic site is present at the N terminus of VP1. However, significant antibody titers to this region of VP1 have been observed in human vaccinees (4a). Thus, major poliovirus B and T epitopes may be located in close physical proximity and similar linkages may be seen with the neutralizing epitopes identified in VP2 and VP3.

Previous studies, using synthetic peptides, have demonstrated that an antipeptide antibody response is enhanced by linkage of T epitopes to this peptide sequence (10, 11, 16, 21). Similarly, studies have shown that neutralizing antigenic sites of other viruses contain both T- and B-cell epitopes (1, 11, 13, 20, 21) and suggest that an efficient antibody response is dependent on the existence of B- and T-cell determinants in close proximity on a single processed antigen fragment. However, the presence of T epitopes within specific neutralizing sites cannot be the sole factor in determining the antigenic spectrum of the humoral response. In mice, immunization with serotype 3 poliovirus strains induces an antibody response to neutralizing site 1 (VP1 region 1091 to 1104) that is immunodominant (14, 22–24). This immunodominant antibody response is not observed upon immunization with serotype 1 poliovirus strains (8, 14, 23, 26). The identification of T epitopes within neutralizing site 1 of a serotype 1 poliovirus indicates that the immunodominance observed in serotype 3 and not serotype 1 strains is not due to the presence or absence of T epitopes within this neutralizing site. Thus, additional factors must contribute to regulation of the relative immunogenicity of specific antigenic sites within the virus.
ACKNOWLEDGMENTS

We thank M. Gefter for the MAb and A. Rogove for the purified capsid proteins.

This work was supported by Public Health Service grant AI122627 from the National Institutes of Health.

REFERENCES


