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Modulation of Humoral Response to a 12-Amino-Acid Site on the Poliovirus Virion†

JOSEPH P. ICENOGLE,[1] PHILIP D. MINOR,[2] MORAG FERGUSON,[2] AND JAMES M. HOGLE[1]*

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037,[1] and National Institute for Biological Standards and Control, London NW3 6RB, United Kingdom[2]

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Most monoclonal antibodies to poliovirus 3 but not poliovirus 1 require a single 12-amino-acid sequence in virion protein VP1 for neutralization (site 1). None of the available monoclonal antibodies requiring this site bound virions after tryptic cleavage of site 1. This result allowed the amount of site 1-specific antibodies to be determined in an antiserum by comparing its reactivity with virus and trypsin-cleaved virus. Antisera to poliovirus 3 Sabin strain (PS3) but not poliovirus 1 Sabin showed site 1 immunodominance, consistent with the frequency of isolation of site 1-specific monoclonal antibodies to these viruses. Cleavage of site 1 prior to immunization dramatically reduced the immunogenicity of this site in PS3. However, the antiserum against trypsin-cleaved PS3 still had a high neutralization titer, demonstrating that sites other than site 1 can elicit a neutralizing response to PS3. Other antisera to PS3 showed significant variability in the response to site 1, indicating that other factors, such as the genetic background of inbred mouse strains, the species immunized, and the immunization protocol, also affect immunodominance. In particular, a serum from a human infant recently immunized with oral trivalent vaccine had little response to site 1.

Poliovirus provides an excellent model for the characterization of the humoral response to viral pathogens. Several strains of the virus (including the Sabin vaccine strains of all three serotypes and the parental neurovirulent strains of poliovirus 1 [Mahoney] and poliovirus 3 [Leon]) have been sequenced (8, 12–15); the three-dimensional structure of the Mahoney strain of poliovirus 1 has recently been determined at high resolution by X-ray crystallographic methods (4), and the neutralization sites of representative strains of all three serotypes of poliovirus have been mapped by sequencing variants which are resistant to neutralizing monoclonal antibodies (1, 2, 9, 9a, 10, 16). The sequence changes in these variants have been located on the three-dimensional structure determined by X-ray crystallographic studies and have been shown to map on four discrete sites on the surface of the virion (5). Recent evidence suggests that two of these sites are linked (9a). Interestingly, the relative importance of each of the three sites in the humoral response of mice to poliovirus appears to be a function of the serotype or strain of the virus or both. Thus, the majority of monoclonal antibodies to the Leon and Sabin strains of poliovirus 3 select for mutations in a 12-amino-acid site in virion protein VP1 (residues 89 to 100), which is part of the site designated site 1, whereas the majority of monoclonal antibodies to the Sabin and Mahoney strains of poliovirus 1 select for mutations in sites 2 and 3, which include residues in VP2 and VP3, respectively.

We previously showed that there is a unique trypsin cleavage site at Lys-99 of VP1 in the Sabin strain of poliovirus 1 (PS1) (3). Cleavage at Lys-99 did not produce a significant decrease in infectivity and was shown not to affect the ability of a rabbit antiserum to neutralize PS1. We have now extended these studies to include seven anti-poliovirus 1 monoclonal antibodies. Of the seven monoclonal antibodies tested, six had immunoprecipitation titers against virus and trypsin-cleaved virus which were indistinguishable. These six monoclonal antibodies induce mutations in sites 2 and 3 (Marie Chow, personal communication). The remaining monoclonal antibody (C3 [16]) had a marked reduction in titer against trypsin-cleaved virus. This antibody (which was raised against heat-treated virions) is the only anti-poliovirus 1 monoclonal antibody which is known to induce mutations in the area of the trypsin cleavage site (R. Crainic, personal communication).

In our earlier communication, we also noted that the Sabin strain of poliovirus 3 (PS3) has a unique trypsin cleavage site in capsid protein VP1 which produced two fragments similar in size to those seen in PS1 (3). By analogy with PS1, we argued that the cleavage site was at Arg-98 of VP1 in PS3. This prediction has now been confirmed by sequencing. The sequence of the amino terminus of the large fragment was Ala-Gln-Lys-Leu-Phe, as expected for residues 99 to 103 of VP1 (14), thus establishing cleavage at Arg-98. There was no detectable leucine in the first sequencing cycle, indicating no detectable cleavage at Lys-101. Although neither the amino nor carboxy termini of the small (amino-terminal) fragment were sequenced, additional cleavages in the small fragment are unlikely, since the last basic residue in this fragment is Arg-81 and since residues 1 to 85 are buried in the interior of the virion (4). Note that PS1 has two additional amino acids very near the N terminus of VP1 and thus, Lys-99 in PS1 is in an equivalent position to Thr-97 in PS3 (14, 15).

Immunoprecipitation titers against virions and trypsin-cleaved virions were also determined for a panel of monoclonal antibodies against poliovirus 3 whose specificities have been previously determined (Table 1). Without exception, antibodies requiring site 1 did not bind to trypsin-cleaved virions. Binding was eliminated even for antibodies which were insensitive to mutations at Arg-98 (monoclonal antibody 134) or Ala-99 (monoclonal antibodies 27-4-4 and 25-1-4). Only one antibody of the panel (monoclonal antibody 138) showed equivalent titers against virions and trypsin-cleaved virions. This monoclonal antibody has been shown to select for mutations in site 3 (specifically at

* Corresponding author.
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TABLE 1. Lack of binding after tryptic cleavage at arginine 98 of monoclonal antibodies requiring amino acids in the region containing residues 89 to 100 of VP1 of PS3

Monoclonal antibody	Amino acids in VP1[a] required for neutralization	Immunoprecipitation titer[b] (10^4 cpm/μl) of [^{3}H]leucine-labeled virions with:	
		All sites intact	Site 1 cleaved
	E V D N E Q P T T R A Q$_{100}$		
134	× × × × × ×	320	<0.04
165	× × × × × × × ×	13	<0.13
175	× × × × × × ×	130	<0.04
194	× × × × × × ×	790	<0.20
197	× × × × × × × ×	40	<0.13
199	× × × × × × × ×	100	<0.04
204	× × × ×	1,300	<0.06
208	× × × × × × × ×	400	<0.04
27-4-4	× × × × × ×	250	<0.13
25-1-14	× × × × ×	32	<0.04
	Y G P G V D Y R N N L D$_{290}$		
138	× × ×	40	40

[a] Amino acids are indicated by the International Union of Pure and Applied Chemistry-International Union of Biochemistry standard single-letter code (7). X's indicate amino acids in the indicated regions of VP1 which are changed in viruses not neutralized by the indicated monoclonal antibodies (9). No viruses with variant amino acids at position 90, 92, or 94 were available. The top sequence corresponds to residues 89 to 100, and the bottom sequence to residues 279 to 290, of VP1 of PS3.

[b] Immunoprecipitation titers were determined by reacting a constant amount of [^{3}H]leucine-labeled antigen with decreasing amounts of monoclonal antibody and determining the amount of antigen in antigen-antibody complexes by immunoabsorption with *Staphylococcus aureus* cells. See Fig. 1 for typical titration curves and a more detailed description of the procedure.

residues 286 to 288 of VP1) and is known to be insensitive to mutations in site 1 (9).

Thus, trypsin cleavage disrupted the ability of all monoclonal antibodies known to require residues 89 to 100 of VP1 to bind to either poliovirus 1 or poliovirus 3. If one assumes that trypsin-cleavage affects antibodies in sera in a similar fashion, the titer of a serum against trypsin-cleaved virions should reflect the precipitating response to all sites on the virus except residues 89 to 100 of VP1. The relative immunoprecipitation titers of a serum against virions and trypsin-cleaved virions can thus be used to estimate the percentage of the precipitating response in the serum which is directed against residues 89 to 100 of VP1.

Hyperimmune sera against PS1 and PS3 were prepared in BALB/c mice. To assess the reproducibility of the experiments, we did three separate immunizations. In each case, four male mice each were immunized intraperitoneally with PS1 and PS3. The sera from the four mice in each experiment were pooled, and the immunoprecipitation titers against virions and trypsin-cleaved virions were determined (Fig. 1 and Table 2). Several precautions were taken in determining these titers. Cleaved virions were derived from virions by trypsin cleavage, ensuring that the two antigens had exactly the same specific activity. Serum volumes necessary for 50% precipitation of antigen were determined with multiple serum dilutions over the range of 0 to 100% antigen precipitation. The titrations against virions and cleaved virions were performed in parallel to normalize for errors in the serum dilutions. Note that although the exact titers varied for the three independent immunizations, the ratios of the titers against virions and cleaved virions were highly reproducible. The simplicity and accuracy of this procedure made it the assay of choice for the analysis of subsequent sera. When neutralization titers against virions and trypsin-cleaved virions were determined with representative monoclonal antibodies and antisera, the titers paralleled those obtained by immunoprecipitation.

Antisera to PS1 had very similar titers against virions and trypsin-cleaved virions (ratio of cleaved/intact virion titers, 0.77 to 0.84). Absorption of one of these sera with cleaved virions produced a marked decrease in both titers (Table 2) but eventually yielded a serum dominated by site 1 antibodies (ratio of cleaved/intact virion titers, 3/20 or 0.15). From this result we concluded that site 1 antibodies constitute only a small portion of the precipitating response to PS1. In contrast, the titers of antisera to PS3 against trypsin-cleaved virions were only 0.07 to 0.13 of those against intact virions. Absorption of one of these sera with cleaved virions dramat-

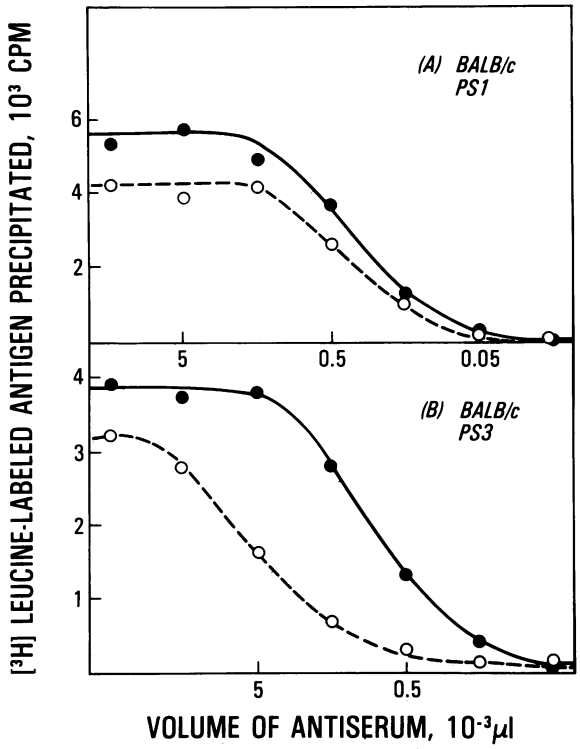

FIG. 1. Representative titration curves for antisera obtained from BALB/c mice immunized with PS1 and PS3. Titrations against virus (●) and trypsin-cleaved virus (○) are shown. About 5 μg of Sabin virions in Dulbecco phosphate-buffered saline containing 0.1% bovine serum albumin was incubated with either 5 μg of trypsin for 24 h at 4°C (PS3) or 25 μg of trypsin for 24 h at room temperature (PS1), producing a single cleavage near residue 100 of VP1 in each virus. Excess trypsin inhibitor from soybean was then added. Immunoprecipitations were carried out in Dulbecco phosphate-buffered saline containing 0.1% bovine serum albumin essentially as previously described (6). Briefly, 50 μl of various dilutions of antiserum was reacted with 50 μl of the indicated radioactive antigens. Sufficient goat antiserum to mouse immunoglobulin G (IgG), IgA, and IgM (lot 0611-3261; Cooper Biomedical, Inc.) to react with any IgM or IgA compelxes was then added and allowed to react. The complexes were absorbed onto *S. aureus* cells and removed by centrifugation. Radioactivity was released by boiling in sodium dodecyl sulfate. Titers were calculated by dividing one-half the amount of input antigen by the microliters of antiserum necessary to precipitate this amount of antigen. The maximum precipitation in each case was equal to the amount of input antigen.

TABLE 2. Immunodominance of site 1 in BALB/c antisera to PS1, PS3, and cleaved PS3

BALB/c antiserum to[a]:	Immunization group	Immunoprecipitation titer[b] (10^4 cpm/μl) of [^{3}H]leucine-labeled virions with:		Ratio of titers	Response to site 1[c]
		All sites intact	Site 1 cleaved		
PS1	1	830 (20)[d]	635 (3.0)[d]	0.77 (0.15)[d]	+
PS1	2	300	253	0.84	±
PS1	3	1,200	1,010	0.84	±
PS3	1	256 (200)[d]	32 (0.4)[d]	0.13 (0.002)[d]	++++
PS3	2	1,100	80	0.07	++++
PS3	3	1,190	101	0.08	++++
Cleaved PS3		40 (1.6)[d]	32 (1.3)[d]	0.80 (0.81)[d]	±

[a] Four 6- to 8-week-old male mice per immunization group were injected intraperitoneally with 6 to 13 μg of antigen in Freund complete adjuvant on weeks 0 and 2. Sera were taken at week 4, pooled, and titrated. All mice were from the Scripps Clinic and Research Foundation breeding colony.

[b] See Fig. 1 for typical immunoprecipitation data.

[c] Ranges for evaluation of the ratio of titers are 0 to 0.2 for ++++, 0.2 to 0.4 for +++, 0.4 to 0.6 for ++, 0.6 to 0.8 for +, and 0.8 to 1.0 for ±. These ranges are about three-fold higher than the variability between experiments.

[d] Titers in parentheses are the titers after absorption of the indicated antiserum with trypsin-cleaved PS1 or PS3 virions. Antiserum was reacted with 3 μg of cleaved virions per μl of antiserum, the complexes were removed by centrifugation, and the antiserum was retitrated. In the case of PS1 antiserum, two such absorption cycles were necessary.

ically reduced the titer against cleaved virions without significantly reducing the titer against intact virions. We therefore concluded that the response to site 1 strongly dominates the precipitating response of BALB/c mice to PS3. These results clearly demonstrated that the difference in the dominance of residues 89 to 100 in VP1 in Mahoney-derived strains of poliovirus 1 and Leon-derived strains of poliovirus 3 which was originally observed in the monoclonal antibody mutation experiments is reflected in sera and is therefore not an artifact of monoclonal antibody production and selection.

There are several possible explanations for the type-specific differences in the dominance of site 1 in mice. First, the dominance of site 1 in poliovirus 3 may reflect the lack of immunogenicity of sites other than residues 89 to 100 of VP1 in Leon-derived strains of poliovirus 3. Second, the difference in dominance may reflect structural differences in poliovirus 1 and poliovirus 3 which cause increased immunogenicity of residues 89 to 100 in Leon-derived strains of poliovirus 3. Third, the dominance may be a reflection of host factors which control the relative immunogenicity of the sites on the viruses.

To assess the immunogenicity of sites other than residues 89 to 100 of VP1 in poliovirus 3, we used trypsin-cleaved PS3 virions to produce hyperimmune serum in BALB/c mice. The serum had a high neutralization titer against PS3 (10^6 PFU/ml of serum). Under identical conditions, the serum to PS3 from immunization group 1 (Table 2) also had a titer of about 10^6 PFU/ml. The relative immunoprecipitation titers against virions and trypsin-cleaved virions, both before and after absorption with cleaved virions, indicated that the response was dominated by sites other than residues 89 to 100 of VP1 (Table 2, row 7). Thus, sites other than residues 89 to 100 of VP1 in PS3 can elicit a strong neutralizing response. In another study, trypsin-cleaved PS3 was used to produce neutralizing monoclonal antibodies. The mutations selected with these antibodies map to sites 2 and 3 in locations similar to those of the mutations observed for poliovirus 1 (9a). Thus, the dominance of site 1 in the response to intact PS3 cannot be ascribed to the lack of immunogenicity of other sites.

The possible role of structural differences in the antigenic sites of poliovirus 1 and poliovirus 3 is more difficult to assess. Comparison of the known sequences of various poliovirus strains shows that the antigenic sites are in areas of sequence divergence. There are, however, no major deletions or insertions which would be expected to lead to marked differences in exposure of the antigenic sites in the two viruses. A more precise assessment of structural differences awaits the completion of structural studies of PS3 and PS1 which are currently in progress (J. M. Hogle, unpublished results).

The role of host factors in the dominance of site 1 was investigated by determining the relative immunoprecipita-

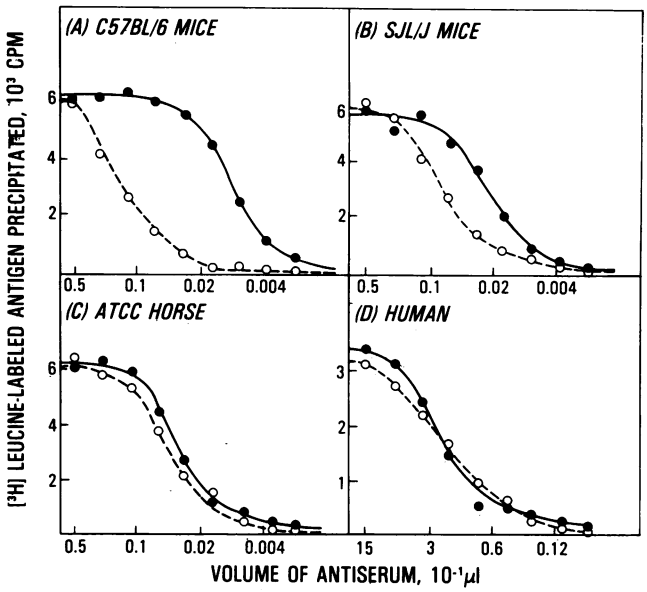

FIG. 2. Immunodominance of residues 89 to 100 of VP1 of PS3 in various antisera. Immunoprecipitations of virus (●) and trypsin-cleaved virus (○) were done as described in the legend to Fig. 1. While the curves for horse and human antisera were obtained without second antibody, the results were unchanged when antisera to human immunoglobulins G, A, and M (Cooper Biomedical, Inc.) or to horse immunoglobulin M (Cooper Biomedical, Inc.), respectively, were included in the assays. ATCC, American Type Culture Collection.

TABLE 3. Immunodominance of site 1 in various antisera

Antiserum produced in:	Virus	Expt	Immunoprecipitation titer[a] (10^4 cpm/μl) of [^{3}H]leucine-labeled virions with:		Ratio of titers	Response to site 1[b]
			All sites intact	Site 1 cleaved		
C57BL/6 mice[c]	PS3	1	310	20	0.06	++++
BALB/c mice[c]	PS3	1	1,100	80	0.07	++++
DBA/2J mice[c]	PS3	1	1,500	253	0.17	++++
C3H/St mice[c]	PS3	1	377	64	0.17	++++
SWR/J mice[c]	PS3	1	2,120	562	0.27	+++
SJL/J mice[c]	PS3	1	130	40	0.30	+++
C57BL/6 mice[c]	PS3	2	320	61	0.19	++++
SJL/J mice[c]	PS3	2	141	62	0.44	++
Horse[d]	Poliovirus 3 Leon strain	1	120	85	0.71	+
Monkey[e]	PS3	1	1,800	1,600	0.88	±
Human[f]	PS3	1	0.76	0.76	1.00	±
C57BL/6 mice[c]	PS1	1	480	350	0.73	+
SJL/J mice[c]	PS1	1	320	250	0.78	+
Monkey[e]	PS1	1	570	500	0.87	±
Human[f]	PS1	1	7.9	7.9	1.00	±

[a] See Fig. 2 for immunoprecipitation data for C57BL/6, SJL/J, horse, and human antisera to PS3.
[b] Evaluations were made as described in Table 2, footnote *c*.
[c] Mice were immunized as described in Table 2, footnote *a*.
[d] Horse antiserum was from the American Type Culture Collection (NIAID V-003-501-560). Poliovirus 3 strain Leon and PS3 have identical amino acid sequences in site 1.
[e] Monkeys were immunized intramuscularly with about 10^8 PFU of virus. For PS1, immunizations were done on weeks 0, 2, and 8, and serum was taken on week 13. For PS3, immunizations were done on weeks 0, 2, 9, and 13, and serum was taken on week 15.
[f] Serum was taken from a 10-month-old healthy male immunized with trivalent oral poliovaccine at 3 and 5 months of age. Significant vaccine-induced immunoglobulin G levels are expected at this stage of a live-poliovirus vaccination protocol (11).

tion titers against cleaved and intact virions in a number of inbred strains of mice and in several outbred species of animals, including horses, monkeys, and humans. Representative titrations are shown in Fig. 2, and the results are summarized in Table 3.

The results with the inbred strains of mice indicated that although residues 89 to 100 of VP1 dominated the response to PS3 in all strains of mice tested, the degree of dominance varied greatly from strain to strain (the ratio of titers varied from 0.06 for C57BL/6 mice to 0.30 for SJL/J mice). The experiment was repeated with C57BL/6 and SJL/J mice (Table 3, rows 7 and 8). This repeat experiment used a second preparation of virus which, for unknown reasons, yields uniformly higher ratios of titers. Nonetheless, the dependence of immunodominance in the response to PS3 on mouse strains was clearly reproduced.

Preliminary experiments with congenic mice, which differ genetically only at the *H-2* locus, did not reveal a linkage of dominance to the *H-2* haplotype (data not shown). Finally, it is of some interest to note that there was little response to either PS1 or PS3 in T-cell-deficient nude BALB/c mice (titers, $<10^4$) (data not shown). This latter result argues that T-cell functions are required for an efficient murine response to poliovirus and suggests that factors involved in the presentation of antigens contribute to the immune response.

In contrast to the results with mice, poliovirus 3 induced a small response to site 1 in other species (horses, monkeys, and humans) (Fig. 2C and D; Table 3, rows 9, 10, and 11). These results may indicate a wide species variation in the immunodominance of site 1 in poliovirus 3. It should be noted, however, that there are other possible explanations for this result. The lack of dominance of site 1 may simply reflect a broader repertoire in the immune response of outbred animals. Alternatively, the differences in the degree of dominance of site 1 in the response to poliovirus 3 may reflect differences in the route of immunization. The mouse sera were all prepared by intraperitoneal injection, while the horse and monkey sera were prepared by intramuscular injection, and the single sample of human sera came from an infant recently vaccinated with the oral trivalent Sabin vaccine. These various modes of immunization may affect antigen stability. Particularly in the case of human sera, where the virus resides primarily in the gut, there is a definite possibility that the antigen which produced the response could have been cleaved virions. Indeed, recent results suggest that the virus isolated from the stools of healthy vaccinees consists primarily of cleaved virions (P. D. Minor, unpublished data).

PS1 induced little response to residues 89 to 100 of VP1 in any of the strains of mice tested. All strains of mice tested with PS3 were also tested with PS1, but only two are listed for brevity (Table 3, rows 12 and 13). Consistent with the observations with PS1 in mice, other species (monkeys and humans) showed only a minor precipitating response to residues 89 to 100 of VP1 in PS1 (Table 3, rows 14 and 15).

In summary, the data presented here confirm and extend the observation made with monoclonal antibodies that a single 12-amino-acid loop (residues 89 to 100 of VP1) dominates the murine immune response to Leon-derived strains of poliovirus 3 but not the Mahoney-derived strains of poliovirus 1. The results also demonstrate that the dominance of this site in poliovirus 3 is not due to a lack of immunogenicity of other sites and that the control of dominance may be complex, including factors such as the species and genetic background of the animal immunized or the route of administration or both. Thus, considerable caution

must be exercised in attempts to extrapolate the mapping of the immune response to viral antigens from data generated from inbred lines of a single species. The results further suggest the possible importance of the proteolytic processing of viral antigens in modulating the fine spectrum of the immune response to viral antigens. These observations may be of particular importance in ongoing attempts to design new attenuated vaccines for poliovirus and related viruses by recombinant DNA methods. Finally, the results suggest that the protecting immune response generated by oral administration of the live PS1 and PS3 vaccines is directed against sites other than residues 89 to 100 of VP1.

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