

Changes in Three of the Four Coat Proteins of Oral Polio Vaccine Strain Derived from Type 1 Poliovirus

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Little is yet known about the nature, or extent, of the changes involved in attenuation of neurovirulent poliovirus. The tryptic comparison reported here, of coat proteins from the Sabin type 1 polio vaccine and parental Mahoney virus, provides a useful approach and affords some insight into this question. The main obstacle, separation of the labile proteins VP1 and VP2 in an intact state from the vaccine strain, was overcome by incorporating 3.5 M urea into an otherwise standard preparative gel electrophoresis system. Tryptic maps revealed six altered leucine-containing peaks: two in VP1, none in VP2, three in VP3, and one in VP4. It is estimated, after correcting for leucine-free peptides, that the coat protein sequences may have undergone some 10 to 13 amino acid replacements, roughly 1.5% of the total, in the course of attenuation leading to the vaccine strain.

Attenuated polioviruses have been used with great success as oral vaccines (7, 29). However, the process by which they were selected is highly empirical (25), and little is yet known about the underlying mechanisms responsible for attenuation. A more complete understanding of attenuation is of interest because the Sabin oral vaccine strains occasionally revert to neurovirulence, causing episodes of paralytic poliomyelitis among vaccine recipients and their contacts (7, 23). The low frequency of reversion suggests that reversion, like attenuation (24, 25), occurs by a series of mutational steps.

One approach towards understanding attenuation, and also reversion to virulence, is to determine at the molecular level the nature of the differences between attenuated and neurovirulent poliovirus strains. Of particular interest in this communication is a report (21) that the VP1 protein of the Sabin type 1 oral vaccine strain, LSc 2ab, differs in electrophoretic mobility from that of its neurovirulent parent, Mahoney. The surfaces of these two strains are distinguishable by serological tests (22, 27). Because VP1 is an important element of the virion surface it is likely to play a key role in recognition of host receptors, an interaction believed important in controlling species specificity and tissue tropism (8).

Attempts to characterize the differences between VP1 of the attenuated type 1 virus and its virulent parent have been impeded by inability to separate VP1 and VP2 of the vaccine strain (21). We report here that urea selectively alters

the relative mobility of these two proteins on sodium dodecyl sulfate (SDS)-polyacrylamide gels. We have exploited this behavior to separate all four coat proteins in the oral vaccine strain to compare their tryptic maps with those of its neurovirulent parent.

MATERIALS AND METHODS

Virus. Mahoney virus, neurovirulent strain M56 of Jonas Salk, was obtained from M. Hatch, Center for Disease Control, Atlanta, Ga. This virus (lot 54) had been passed four times in monkey kidney culture at Connaught Laboratories, Toronto, Canada, twice again in monkey kidney culture, and once in human aminionic (FL) cells at the Center for Disease Control. Oral vaccine strain LSc 2ab (Sabin type 1) was sent to us by E. Seligmann, Jr., Bureau of Biologics, U.S. Food and Drug Administration, Bethesda, Md. High-titer inoculation stocks were prepared in suspension culture after three successive plaque purifications of reference seed.

Cells. Virus was propagated in H-HeLa cells grown in medium B (20) containing 10% bovine serum.

Preparation of radiolabeled poliovirions. HeLa cells were infected in suspension culture with poliovirus (50 PFU/cell) by the procedures of McGregor et al. (19), except that the attachment period was 30 min. Poliovirions were labeled by addition at 2.0 h postinfection of L-[U-¹⁴C]leucine (Amersham-Searle; CFB.67) to Mahoney-infected cultures and L-[4,5-³H]leucine (Amersham-Searle TRK.170) to LSc 2ab-infected cells. Incubation in the presence of label continued at 37°C until 6.5 h postinfection, when the infected cells were sedimented by low-speed centrifugation.

Cell pellets were suspended in unlabeled medium AL (19), and cell-associated virus (approximately 70% of total plaque formers) was released by three freeze-thaw cycles. Cell debris was removed by centrifugation, and the virus was pelleted through a cushion of

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30% sucrose in 1 M sodium chloride, 0.02 M Tris-acetate (pH 7.5), and 1% bovine serum albumin. Virus pellets were suspended in phosphate-buffered saline (11) containing 0.1% bovine serum albumin and clarified by centrifugation for 5 min at $12,000 \times g$.

The virus-containing supernatant was diluted to 4.8 ml with phosphate-buffered saline–0.1% bovine serum albumin and brought to a density of 1.34 g/ml (31.4%, wt/wt) by dissolving 2.2 g of crystalline cesium chloride. Virions were resolved from empty capsids by isopycnic banding in cesium chloride gradients formed after 18 h of centrifugation at 6°C at 45,000 rpm in a Spinco SW50 rotor. Gradients were fractionated by bottom puncture of the centrifuge tube. Virion fractions were pooled, diluted into 0.01 M sodium phosphate (pH 7.2), and pelleted through a 30% sucrose cushion containing the same buffer. Virus pellets were suspended in 0.01 M sodium phosphate (pH 7.2) and stored at –70°C. The recovery of input radiolabel in purified Mahoney virions was 4.0% (0.5 mCi of [¹⁴C]leucine per 4×10^8 infected cells) and 3.1% (5.0 mCi of [³H]leucine per 2×10^8 infected cells) in purified LSc 2ab virions.

Polyacrylamide gel electrophoresis. Conditions for SDS-polyacrylamide gel electrophoresis follow the procedures of Medappa et al. (20). Gels contained 9.8% acrylamide, 0.1% *N,N,N',N'*-tetramethylethylenediamine, 0.1% SDS (Fisher lot 726579), 0.1 M sodium phosphate (pH 7.2), cross-linker (0.3%, vol/vol, ethylene diacrylate for analytical gels; 0.2%, wt/vol, *N,N'*-methylenebisacrylamide for preparative gels and transverse urea gradient gels), and the indicated concentrations of urea. Polymerization was catalyzed by addition of ammonium persulfate to a final concentration of 0.05% (0.5 M urea analytical gels) or 0.025% (3.5 M urea preparative gels; transverse urea gradient gels). The electrode buffer, 0.1 M sodium phosphate (pH 7.2) containing 0.1% SDS and 0.05 M neutralized 3-mercaptopropionic acid, was circulated between upper and lower buffer vessels. Samples for electrophoresis were adjusted to 1% SDS, 0.5 M urea, 0.1% 2-mercaptoethanol, 10% sucrose and 0.01% bromophenol blue and heated at 100°C for 5 min prior to application to the gels.

Procedures for fractionation and analysis of SDS-polyacrylamide gels with a Gilson automatic fractionator have been described (20). Preparative gels (1.2 by 25 cm) were fractionated in the same way by using a specially built plunger and processing head which crushed 1-mm segments of the gel and delivered the slurry in 0.35 ml of elution buffer (0.01 M sodium phosphate, pH 7.2, 0.1% SDS, 0.05% 2-mercaptoethanol, 1 mM EDTA). Fractions were collected into bovine serum albumin-coated 2-ml flat-bottomed plastic beakers (Scientific Products catalog no. B2714-1). After 24 h of incubation at room temperature, the distribution of radioactivity was determined by taking 10- μ l samples from each fraction. Peak fractions were pooled by centrifugation ($600 \times g$, 5 min) through Surety filtration columns (Evergreen Scientific, catalog no. 3045) attached to the tops of 3.5-ml plastic beakers (Evergreen Scientific, catalog no. 1008). Pools were stored at –70°C.

Transverse urea gradient gels. Transverse urea gradient gels were cast between glass plates into poly-

acrylamide slabs (15 by 15 by 0.15 cm). Glass plates were assembled with a petrolatum-sealed spacer placed along one side and temporary spacers placed 15 cm apart at the top (notched edge side) and bottom of the plates. The temporary spacers were sealed with 1.2% agarose containing 0.1% SDS. The assembled plates were clamped with steel binder clips and set with the open edge up, notched edge to one side. Linear 0 to 8 M urea gradients were formed by use of an open two-chamber mixer in which the reservoir contained 17.5 ml of monomer lacking urea and the mixing chamber contained an equivalent volume of monomer containing 8 M urea. The gradient was delivered at a rate of 8 ml/min by using a peristaltic pump. After polymerization, the temporary spacers were removed, a petrolatum-sealed spacer was inserted into the open side adjacent to the notched edge, and the resultant space formed at the bottom was filled with electrolyte agarose (0.01 M sodium phosphate, pH 7.2, 0.01% SDS, 1.2% agarose). Slabs were placed upright into the electrophoresis unit. Pre-electrophoresis was for 60 min at 3.0 V/cm. Samples (0.5 ml) were then layered under the electrode buffer across the entire top of the gel. After electrophoresis (3.0 V/cm, 38 mA, 16 h), radioactivity was detected by fluorography (4) using preflashed (16) Kodak RP Royal X-Omat (XR-5) X-ray film.

Tryptic peptide analysis. Differentially labeled pairs of homologous polypeptides, isolated on polyacrylamide gels, were mixed (³H: 27 dpm/dalton; ¹⁴C: 2.5 dpm/dalton), and the samples in elution buffer were adjusted to pH 8 by addition of 0.1 volume of 0.5 M Tris-hydrochloride (pH 8.0). Bovine serum albumin was added to 1 mg/ml as a carrier. After reduction in the presence of dithiothreitol at 3.5 mg/ml (37°C, 30 min) and alkylation with iodoacetamide at a final concentration of 9 mg/ml (0°C, 60 min), the proteins were precipitated by adding an equal volume of 50% (wt/vol) trichloroacetic acid. The precipitate was washed three times with 5 ml of ether to remove residual acid and then dispersed in 1.0 ml of 0.1 M NH₄HCO₃ (pH 8.0). Digestion was at 37°C for 16 h with tosyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington), with a mass ratio of trypsin to bovine serum albumin of 1:25, and for 8 h more with a second, equivalent sample of trypsin. The digest was lyophilized, and the residues were dissolved in 1 ml of 0.2 M pyridine acetate at pH 3.1, filtered through a 0.45- μ m membrane filter, and applied to a column (0.6 by 48 cm) of Technicon Chromobeads type P cation exchange resin. The high-pressure chromatogram (12) was developed at 53°C with a linear gradient of pyridine acetate increasing in pH and concentration (0.2 M, pH 3.1 to 2 M, pH 5.0) for the first 200 fractions. The column was washed with 2.0 M pyridine acetate (pH 5.0) for the final 40 fractions. Fractions (1.5 ml) were collected at 3-min intervals into scintillation vials, lyophilized, and assayed for radioactivity.

RESULTS

Differential effect of urea on the electrophoretic mobility of coat protein from Mahoney virus and its attenuated derivative LSc 2ab. Figure 1A compares the electrophoretic profiles of proteins from Mahoney and the

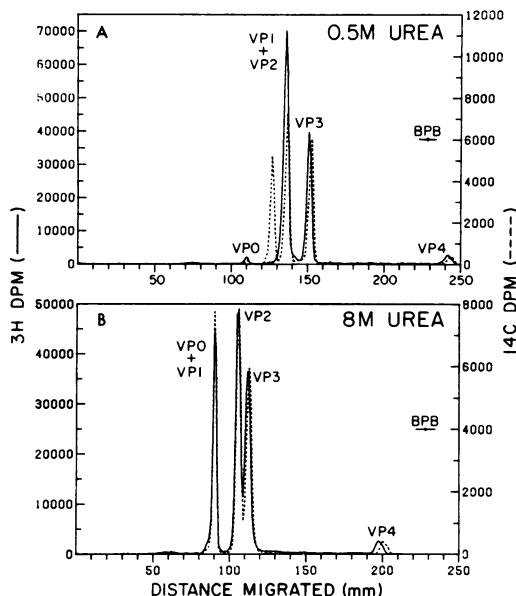


FIG. 1. Relative electrophoretic mobilities of the virion proteins of LSc 2ab (solid line) and Mahoney (dashed line) in the presence of low (A) and high (B) concentrations of urea. Identical samples containing disrupted radiolabeled virions of LSc 2ab ($690,000^3\text{H dpm}$) and Mahoney ($88,000^{14}\text{C dpm}$) were applied to ethylene diacrylate-cross-linked polyacrylamide gels (0.6 by 25 cm) containing either 0.5 or 8 M urea. Electrophoresis was performed at a current of 8 mA per gel until the bromophenol blue (BPB) tracking dye band had migrated 23 to 24 cm. The bars indicate the position of the dye band at the end of each run. The 8 M urea gels were run at lower potentials and for longer times (3.0 V/cm, 25 h) than were the 0.5 M urea gels (3.8 V/cm, 20 h). Gels were fractionated and counted as described in the text.

Sabin oral vaccine strain on phosphate-buffered gels containing 0.5 M urea. The Mahoney profile (dashed line) exhibits a typical polioviral pattern: three closely spaced central peaks, corresponding to the three largest major capsid proteins, VP1, VP2, and VP3, plus a smaller, rapidly migrating peak representing the smallest capsid protein, VP4. The small peak, VP0, represents residual precursor proteins, roughly two copies per virion, which fail to be cleaved to VP2 plus VP4 during the viral maturation step of morphogenesis.

The same proteins are found in the LSc 2ab virions (solid line). However, one of the major proteins, VP1, is obscured because it comigrates with VP2 in SDS-phosphate gels at this low urea concentration. This was first demonstrated by Milstien et al. (21), who showed that the two proteins could be separated by electrophoresis in the discontinuous gel system of Laemmli (14). However, we found this system unsatisfactory

for preparative purposes because of an unexplained and poorly reproducible tendency toward degradation of VP1. The effect was selective for VP1 and was also strain specific. Thus VP1 of Mahoney was relatively stable under these same conditions (data not shown). These observations point up a potential pitfall not only in determining polypeptide stoichiometry but also in isolating polypeptide chains sufficiently pure for tryptic comparison.

In seeking alternative ways to resolve the comigrating VP1 and VP2 chains, we found that increasing the urea concentration from 0.5 to 8 M caused marked changes in the electrophoretic profile (Fig. 1B). The effect is particularly striking with the vaccine strain in which VP1 is now well separated from VP2 and comigrates instead with its counterpart in Mahoney virions. Moreover, there was no evidence of proteolysis under these conditions.

Urea concentration for optimal resolution of polioviral chains. Urea-induced changes in protein mobility were not confined to VP1. Thus with 8 M urea, VP0 and VP2 were shifted relative to VP1 and VP3 such that VP1 comigrated with VP0. This suggested that separation might be still further improved at some urea concentration intermediate between 0.5 and 8 M. To examine this possibility more systematically, disrupted virions were layered in a band across the top of a polyacrylamide slab gel containing a transverse linear gradient of urea ranging in concentration from 0 M on the left to 8 M on the right. After electrophoresis, the radiolabeled proteins were detected by fluorography (Fig. 2). The main effect of increasing urea was to gradually decrease mobilities; the slight downward bowing at the extreme left may reflect thermal gradients established across the slabs during electrophoresis. On the average, proteins ran 1.5 times faster in the absence of urea than in 8 M urea. This correlates roughly with the 1.7-fold ratio between the viscosities of 0 and 8 M urea (13). However, the responses of individual polypeptides to increasing urea concentrations were not identical. For example, the mobilities of VP2 and its precursor VP0 were not as greatly reduced as were those of VP1 and VP3. As expected, the greatest reduction in mobility was seen with the VP1 of LSc 2ab, which migrated with VP2 in low urea and with VP0 at urea concentrations above 6 M. While the mechanisms of the differential effects of urea upon polypeptide mobilities are not understood, the transverse urea gradient technique is useful in determining which urea concentrations allow optimal resolution of polypeptides. It is apparent from the patterns in Fig. 2 that the best separations occurred in the region of 3 to 4 M urea.

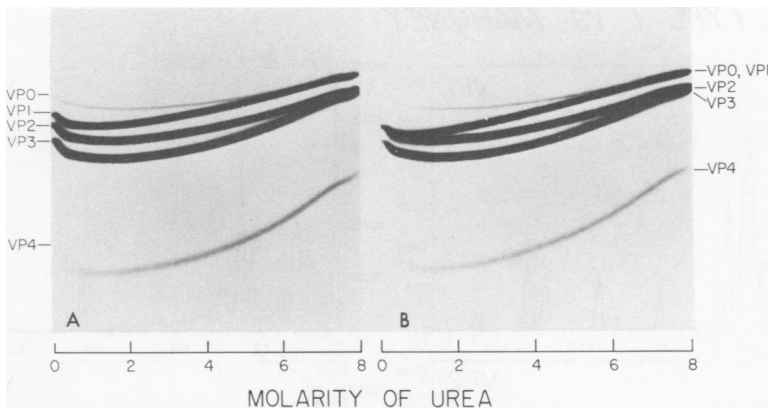


FIG. 2. Effects of varying the urea concentration on the mobilities of Mahoney (A) and LSc 2ab (B) virion proteins shown by transverse urea gradient slab gels. Polyacrylamide slabs (15 by 15 by 0.15 cm) were cast to contain a linear 0 to 8 M urea gradient perpendicular to the direction of electrophoresis. After polymerization, the slabs were rotated 90° for electrophoresis. Samples (0.5 ml) of disrupted Mahoney (88,000 ^{14}C dpm) or LSc 2ab (690,000 ^3H dpm) virions were layered across the top of each gel. Electrophoresis was for 16 h at 3.0 V/cm (38 mA). The positions of the radiolabeled viral proteins were visualized by fluorography (4, 16).

Isolation of proteins and comparison by tryptic mapping. The radioactivity profile of a preparative gel run at the optimum urea concentration, 3.5 M, revealed excellent separation of all coat proteins (Fig. 3). The low background in the region between VP3 and VP4 reveals evidence of only slight proteolysis in the vaccine strain (Fig. 3B) and none in the Mahoney strain (Fig. 3A). Peak fractions, containing the radiochemically pure capsid chains VP1, 2, 3, and 4, were then isolated, and the homologous chains of Mahoney and LSc 2ab were compared by tryptic mapping. To this end differentially labeled pairs of chains were mixed, digested to completion with trypsin, and chromatographed with a pyridine acetate gradient on a sulfonated ion exchange resin (Fig. 4). Acidic peptides, which are uncharged in the starting buffer (0.2 M pyridine acetate, pH 3.1), bind poorly to the resin; they emerge in the early fractions (3 through 8) as a "flow-through" peak. Most of the leucine-containing peptides, however, were retarded, with the most basic ones eluting last. The sensitivity and reliability of the comparisons was enhanced by double-label analysis; thus differences in elution position corresponding to 0.5 fraction could be detected with confidence.

At least six differences (asterisks in Fig. 4) are evident in the tryptic profiles: two in VP1, none in VP2, three in VP3, and one in VP4. In the case of VP1, the vaccine profile (solid line) displayed one peak (12*) not observed in the parental Mahoney strain (dotted line). This extra peak is inconsistent with the presence of a deletion in the vaccine strain and might even be construed as evidence of inserted sequences. More likely, however, either the Mahoney peak 13 is a mixture of two overlapping peptides, or

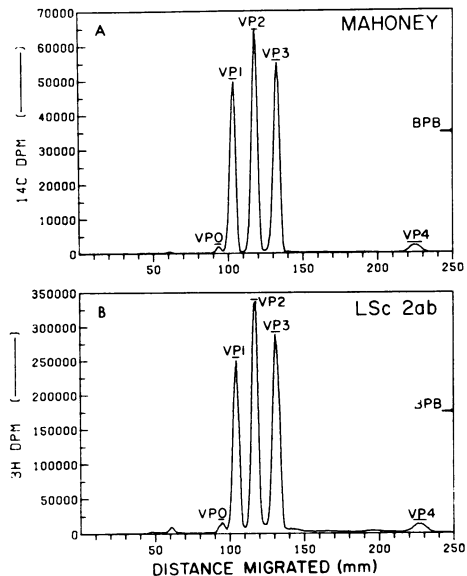


FIG. 3. Purification of Mahoney (A) and LSc 2ab (B) virion proteins on polyacrylamide gels containing 3.5 M urea. Radiolabeled virions of Mahoney (2.0×10^7 ^{14}C dpm) and LSc 2ab (1.5×10^8 ^3H dpm) were disrupted, and each sample was applied to a preparative gel (1.2 by 25 cm). Electrophoresis was at 75 V (24 mA) until the midpoint of the bromophenol blue (BPB) band had migrated 25 cm, about 27 h. The gel was fractionated as crushed 1-mm segments, and the proteins were eluted from the gel as described in the text. The distribution of radioactivity was determined from 10- μl samples from each fraction. Bars above peaks indicate pooled fractions.

VP1 of the vaccine strain contains an additional cleavage site generated by substitution of a lysine or arginine residue.

SABIN TYPE 1 vs. MAHONEY

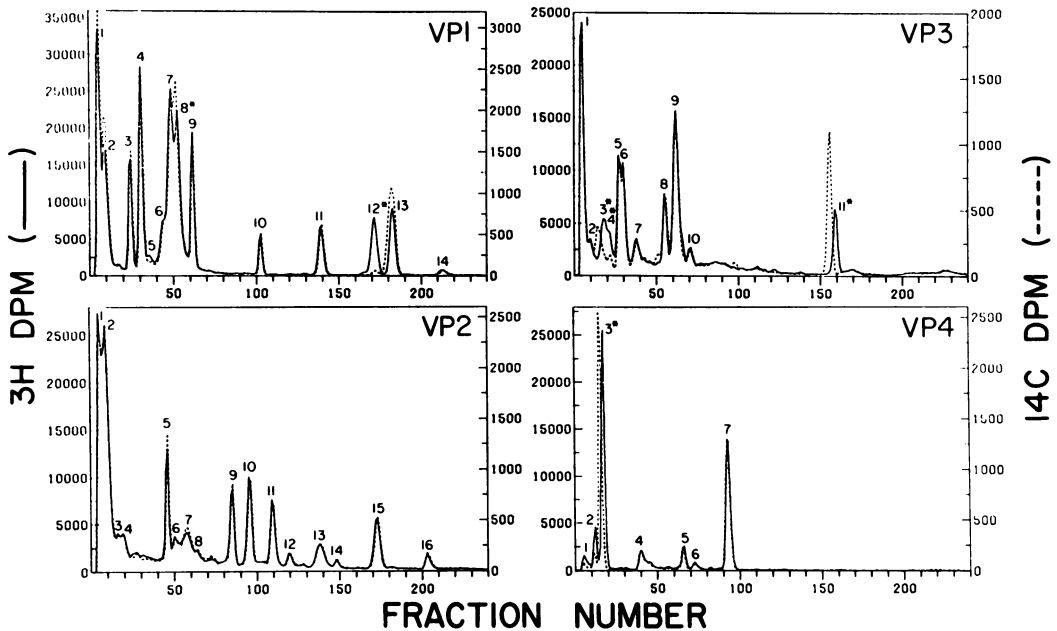


FIG. 4. Tryptic comparisons of the capsid proteins of Mahoney and LSc 2ab. Homologous capsid proteins, purified on polyacrylamide gels as described in Fig. 3, were mixed and digested with trypsin, and the peptides were resolved by cation-exchange chromatography. LSc 2ab, ^3H label (solid line); Mahoney, ^{14}C label (dashed line). The numbers refer to peaks in the vaccine strain; asterisks indicate peptides that differ between the two strains. Recoveries of label (^3H , ^{14}C) from prefiltered tryptic digests were: VP1, 82%, 81%; VP2, 78%, 79%; VP3, 82%, 78%; VP4, 81%, 90%.

Not all peaks are of equal size; this is probably due to differences in the leucine content of the various tryptic peptides and possibly also to traces of chymotryptic fragments in the limit digest. Moreover, leucine-free peptides in digests of protein labeled with radioactive leucine would not have been detected at all by our radioactivity measurements; we estimate the proportion at about 40%. Thus the fraction, P_0 , of such leucine-free peptides is expected, if we assume a Poisson distribution, to be e^{-n} , where n is the average number of leucine residues per tryptic peptide. From the amino acid composition (30), one computes that each of the 60 96,000-dalton protein subunits contains 75 leucines and 42 arginines plus 43 lysines. Because the latter two residues are target sites for trypsin, the coat protein would be expected to generate a total of some 89 distinct tryptic peptides (Table 1). Each tryptic peptide should therefore contain, on average, 75/90 or 0.83 leucine residues. The fraction of tryptic fragments containing zero leucine residues should then be about $e^{-0.83}$ or 43%, and the balance, 57%, should have been positive for leucine.

Thus, of the anticipated 90 tryptic peptides, 57%, corresponding to some 51 peaks, might be

TABLE 1. Expected distribution of leucine-containing tryptic peptides in type 1 polioviral protein

Protein	Leucine residues (L) ^a	Tryptic peptides (T) ^b	Expected fractions of unlabeled peptides ^c	No. of leucine-labeled tryptic peptides	
				Expected ^d	Found ^e
VP1	23	33	0.50	17	14
VP2	23	23	0.37	14	16
VP3	21	22	0.38	14	11
VP4	8	12	0.51	6	7
Total	75	90	0.43	51	48

^a From Wouters and Vandekerckhove (30).

^b (Arg + Lys) + (1 for each protein).

^c Fraction of leucine-free peptides calculated as the P_0 class of the Poisson distribution $P_0 = e^{-n}$, where n ($= L/T$) is the average number of leucine residues per tryptic peptide. For example, for VP1, $P_0 = e^{-23/33} = 0.50$.

^d Fraction of labeled peptides = $1 - [$ fraction of unlabeled peptides (e^{-n})]. Thus the expected number of leucine-labeled peptides is $(1 - e^{-n})$ times the total number of tryptic peptides (T).

^e From Fig. 4.

expected to contain leucine. The number actually observed, 48, is in reasonably good agreement with this value. The same conclusions

apply to more detailed consideration of the individual chains (Table 1).

DISCUSSION

Are surface changes important in attenuation of poliovirus? We have found multiple changes in VP1 and VP3 and one in VP4. If, as originally proposed by Sabin (24), virulence in poliovirus is determined by specific interactions of the virion with sites unique to the target cell, then attenuation may consist of selection for changes in the virion surface.

There is abundant evidence that VP1 contributes importantly to the surface of the virion. Thus, of all four coat proteins VP1 is reportedly the most heavily labeled when intact virions are treated with reagents such as iodine (2, 17), acetic anhydride (17), or *N*-succinimidyl propionate (28). Similar labeling patterns are observed with many other picornaviruses (5, 15, 17, 18). Antibody blocking experiments with mengovirus (18) and protease susceptibility studies with foot-and-mouth disease virus (6) support the idea that, with these viruses also, VP1 participates importantly in virus-cell interactions leading to infection.

The finding of changes in VP3 and VP4 does not necessarily undermine the idea that it is chiefly the virion surface that is altered during attenuation. Recent findings show that VP3 lies in close proximity to VP1. Thus a cross-linked VP1-VP3 complex is the major product formed by treating intact poliovirions with bifunctional reagents such as dimethyl suberimidate (28), dimethyl adipimidate (28), or *bis*-2-[(succinidoxycarbonyloxy)ethyl] sulfone (R. E. Duke, Ph.D. thesis, University of Wisconsin, Madison, 1979). More direct evidence that VP3 contributes to the virion surface comes from a recent report that nearly 60% of the bound label was found in VP3 when intact poliovirions were derivatized with radioactive Bolton-Hunter reagent (28).

Protein VP4, on the other hand, appears not to be derivatized by surface-labeling techniques (2, 5, 15, 17, 18, 28). In this case it can be argued that the difference between the attenuated and nonattenuated virus VP4 is incidental to the attenuation process. Indeed, unpublished studies by one of us (O.M.K.), comparing attenuated and virulent strains of all three serotypes, indicate that the electrophoretic mobility of VP4 on SDS-gels is much less variable than that of VP1 or VP3. Preliminary studies on a type 3 pair, Sabin type 3 (Leon 12a,b) and Leon, also reveals multiple changes in VP1 and VP3 but none in VP2 or VP4. These limited observations suggest that attenuation is accompanied chiefly by changes in VP1 and VP3.

Nature of the changes in VP1 and VP3. We have shown that the difference in mobility between VP1 of the oral vaccine virus and that of its virulent parent disappears as the urea concentration in the SDS-polyacrylamide gel is increased. This behavior, together with the lack of evidence for missing peptides in VP1 in the vaccine strain, suggests that the different electrophoretic behavior of the two VP1 chains is due, not to a deletion, but to relatively minor changes, probably several amino acid substitutions.

It might seem surprising that a few amino acid substitutions could cause a 7% mobility shift in VP1 (corresponding to a 13% change in apparent molecular weight from 35,000 in Mahoney to 31,000 in the vaccine strain), whereas a similar number of substitutions in VP3 causes only a slight shift. A possible explanation is suggested by a recent report that substituting a single hydrophilic residue (glutamine) by a hydrophobic one (leucine) can increase the electrophoretic mobility of a 20,000-dalton protein by as much as 3% (10). This observation suggests that the magnitude of an electrophoretic shift is determined not so much by the number of substitutions as by the net change in hydrophobicity accompanying replacement of the amino acids.

The influence of urea in altering relative mobility of proteins was first reported by Vande Woude and Bachrach (26). Because urea is known to facilitate release of SDS molecules from proteins (9), it seems likely that its effect involves differential release of negatively charged SDS molecules. Differential increases in Stokes radius, as urea completes denaturation (1) analogous to "melting" of hydrophobic interactions between residues in the polypeptide chain, may also be involved.

Estimated number of amino acid substitutions. We have found evidence for six changes in the coat protein of Sabin type 1 oral vaccine derived from Mahoney virus. This is likely to be an underestimate of the true number of substitutions because only about 57% of the peptides could be expected to contain leucine (Table 1). Correcting for such undetected leucine-free fragments suggests that the number of altered tryptic peptides is more nearly 6/0.6 or 10.

Additional peptides may have been overlooked because of insolubility in starting buffer. Up to 20% of the peptides might have been lost in this way (see legend to Fig. 4); then the number of altered peptides might be as high as 10/0.8 or 13. Accordingly, we estimate that the number of changes accompanying attenuation of Mahoney virus to form the Sabin type 1 oral vaccine strain is on the order of 10 to 13, roughly 1.5% of the total number (about 870) of amino

acid residues in the subunit of polioviral coat protein. The presence of so many substitutions seems reasonable considering that the vaccine was derived by a series of more than 100 passages through different tissues (25).

Do noncapsid proteins play a role in neurovirulence? The relatively large number of substitutions in coat protein raises the important question of which are relevant to virulence and whether changes have not also occurred in noncapsid proteins. Indeed it is quite possible that the biochemical determinants of attenuation are not restricted to the coat protein, which represents only about 40% of the genome (23a). On the basis of recombination studies, Bengtsson (3) proposed that attenuation of LSc 2ab may be due to multigenic changes. Some of these changes may occur in noncapsid proteins. Indeed, electrophoretic studies reveal that LSc 2ab and Mahoney also differ in noncapsid proteins 4, 7c, 9a, and 10 (Kew and Rueckert, manuscript in preparation). Detailed tryptic comparisons between corresponding noncapsid proteins await more complete characterization of the relationships among the many nonvirion proteins produced in poliovirus-infected cells (23a).

Defining the molecular basis of neurovirulence in poliovirus. As noted above, the differences described here between Mahoney and the oral vaccine strain are not necessarily all related to the attenuation process. Another limitation is uncertainty about the exact relationship of the Mahoney strain studied here to that used by Sabin to generate the vaccine strain. Clearly future studies directed to defining the molecular basis of neurovirulence in poliovirus should direct more careful attention to genetic relationships and to defining the rate at which poliovirus mutates.

In this regard it would now be of major interest to collect a series of neurovirulent isolates resulting from independent mutations of a well-characterized vaccine strain. Such neurovirulent isolates could then be similarly analyzed for variations in structure with the hope of correlating reversion to neurovirulence with alteration of specific proteins. The realization that amino acid substitutions are detectable by electrophoretic mobility on SDS-polyacrylamide gels suggests that this technique, carefully applied, may be a very useful tool in screening isolates for such altered proteins.

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