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Identification of LSc2ab Strain of Type 1 Poliovirus by Inhibition With Horse Serum and Dextran Sulfate.* (31368)

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Means are now available to distinguish the LSc2ab attenuated virus from a limited number of other type 1 strains of poliovirus provided that their characteristics are known. However, genetically determined markers of the LSc2ab virus (Sabin type 1 vaccine) that will distinguish it from all other type 1 strains, especially wild strains, are still being sought. Most characteristics of LSc2ab are either not specific (*e.g.*, replicative capacity at 40°C) or are too unstable on passage of the virus to differentiate it with certainty (*e.g.*, intratypic immunologic properties). In order to provide a reliable means of distinguishing the LSc2ab virus and its subpassages from other type 1 viruses, whether attenuated or virulent, we have tested the use of two different antiviral substances in combination(1-2). Each inhibitor has a greater effect on LSc2ab than on other type 1 strains; both inhibitors combined have an additive anti-LSc2ab effect. Moreover, mutants resistant to one inhibitor (equine serum) are rare, while mutants resistant to the other inhibitor (dextran sulfate), although rather frequent, appear to retain their sensitivity to the equine serum.

Materials and methods. Cell cultures and media. Primary Rhesus monkey kidney (MK) cells were grown in 60 mm plastic petri dishes with Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS) non-inhibitory for poliovirus, penicillin (100 I.U./ml), streptomycin (100 μg/ml) and nystatin (20 μg/ml) in a humidified atmosphere of 5% CO_2 and air.

Viruses. All strains were type 1. Pools of the attenuated CHAT and LSc2ab vaccines were stored in small aliquots at −20°C for use without further passage. Mahoney virus was prepared by infecting MK cells at an input multiplicity of 3 - 10 plaque-forming units (PFU); the virus-containing medium (MEM) was havested after 24 hours, centrifuged at 2000 rpm and the supernatant kept at −20°C.

The wild type 1 strains, previously described(3), were isolated in different parts of the United States and Puerto Rico from asymptomatic persons and patients with poliomyelitis and had been passed 3 to 4 times in MK cells.† The strains received a further mass passage but were never plaque-passed.

First human-passage strains of the CHAT and LSc2ab vaccines were obtained by vaccination of infants in isolated bassinets. Ten per cent suspensions of feces treated with antibiotics and centrifuged at a low speed were regarded as undiluted human-passage material. After dilution of the supernatants their virus content and characteristics in plaque reduction tests were examined directly without an intermediate passage in cell culture.

Plaque Assay. Duplicate monolayers of MK cells were inoculated with 0.2 ml of virus and placed at 37°C for 60 minutes. The cell cultures were then overlaid with 5 ml of medium consisting of liquefied 1% Noble's agar, 0.055% bovine albumin, 2.5% inactivated FCS, penicillin, streptomycin and nystatin. After 49 hours at 37°C a second overlay with 0.01% neutral red was added,

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† Strains courtesy of Dr. James Nakano.

and plaques were counted on the third and fourth days.

Plaque reduction tests for detection of genetic markers. a) *inhibitory equine serum*: A selected normal equine serum (H_3), inactivated at 56°C for 30 minutes, was incorporated in a concentration of 1.5% in the first overlay of plaque assays(1). Virus sensitive to inhibition was termed ho^- and nonsensitive virus, ho^+(4).

b) *dextran sulfate*: (Pharmacia) molecular weight 2×10^6, was incorporated in the first overlay of plaque assays in a concentration of 100 μg/ml. Sensitive virus was termed m^- and nonsensitive virus m^+(5).

Companion assays of the virus under test were performed by omission of the inhibitors and inclusion of 2.5% noninhibitory FCS in the overlays.

Type 1 strains known to be sensitive (−), partly sensitive (±), or nonsensitive(+) to or enhanced (+) by the inhibitors were included in each test. The properties of the strains were: LSc2ab (m^-, ho^-), CHAT (m^+, $ho\pm$) and Mahoney (m^+, $ho\pm$). The properties of the strains with the inhibitors used in combination were: LSc2ab (m, ho)$^-$ and CHAT and Mahoney (m, ho)$^+$(1).

Plaque reduction was expressed by decreased plaque size and by decreased titer. Reduction in terms of decreased plaque size was calculated by the formula,

$$PR_D\ (\%) = \frac{D_c - D_i}{D_c} \times 100$$

in which D is plaque diameter (mm), c, control without inhibitors and i, inhibitors. The diameters of 10 plaques on two 60 mm monolayers inoculated with 20-40 PFU each were averaged for the calculation of PR_D. Viruses were regarded as sensitive if the PR_D was 90 or more and partly sensitive if the PR_D was between 75 and 90.

Plaque reduction as expressed by decreases in titer (PR_T (%)) was calculated with an analogous formula in which the numbers rather the diameters of plaques were the factors. Pinpoint plaques (0.5 mm in diameter), usually less than a tenth of the size of control plaques, were ignored in the calculation of titers. A PR_T of 75 or more was considered to indicate significant inhibition.

TABLE I. Effect of Human Passage on Inhibition of LSc2ab Virus by Dextran Sulfate.

Virus	Control	Dextran sulfate in overlay (μg/ml) 100	200	500
LSc2ab (unpassed vaccine)	5.4*	3.1	3.0	2.8
LSc2ab (human passage (B341))	4.4	3.1	2.9	3.1

* $\log_{10}$ PFU/ml.

Selection of plaques. Unpassed LSc2ab vaccine (Lederle) was plaque-assayed with standard overlay. Material from the center of single plaques was aspirated with a Pasteur pipette, placed in 1.8 ml Hanks' balanced salt solution (BSS) and frozen. After dilution the virus in each plaque was assayed and characterized directly in plaque reduction tests.

Results. Effect of dextran sulfate. Dextran sulfate alone is a potent inhibitor of the LSc2ab virus. Added to the overlay of a plaque assay of the LSc2ab strain, dextran sulfate reduced the titer of the vaccine from $10^{5.4}$ to $10^{3.1}$ PFU (PR_T 99.5) (Table I). However, even at the virus dilution at which inhibition was effective, one or two full-sized plaques per 100 PFU plated usually appeared despite the presence of dextran sulfate. Moreover, after passage through the human intestine the LSc2ab virus was less sensitive to dextran sulfate, the titer of the passage virus being reduced only from $10^{4.4}$ to $10^{3.1}$ (PR_T 94.9) (Table I). The number of plaques resisting inhibition at the terminal dilution was increased to 3 or 4 per 100. Increases in the concentration of dextran sulfate from 100 to 200 and 500 μg/ml gave less than a 2-fold further reduction in plaque counts and did not reduce the number of resistant plaques (Table I).

A clonal analysis of LSc2ab vaccine, based on testing of the sensitivity of virus in 15 plaques to inhibition by dextran sulfate, disclosed 2 virus populations differing in sensitivity to the inhibitor (Table II). Fourteen of the 15 plaques contained virus more sensitive to inhibition (PR_T of >99.9 to 99.8) than the unplaqued parental virus (PR_T 99.4). In

TABLE II. Analysis of Sensitivity of LSc2ab Virus to Dextran Sulfate.

LSc2ab plaque	Titer of virus in plaque (control)	Virus titer in presence of dextran sulfate (100 μg/ml)	Reduction in titer† ($\log_{10}$)	PR_T (%)
1	18×10^{3}*	0×10*	>3.3	>99.9
2	17×10^{4}	3.5×10	3.7	>99.9
3	54×10^{3}	2×10	3.4	>99.9
4	62×10^{3}	0×10	>3.8	>99.9
5	42×10^{3}	2×10	3.3	>99.9
6	32×10^{3}	4×10	2.9	99.9
7	42×10^{3}	6×10	2.8	99.9
8	23×10^{3}	4×10	2.8	99.9
9	10×10^{4}	22×10^{2}	1.7	97.8
10	60×10^{3}	11×10	2.7	99.8
11	60×10^{3}	7×10	2.9	99.9
12	58×10^{3}	3×10	3.3	>99.9
13	74×10^{3}	3×10	3.4	>99.9
14	69×10^{3}	9×10	2.9	99.9
15	30×10^{3}	1×10	3.5	>99.9
LSc2ab, unplaqued	43×10^{3}	23×10	2.3	99.4
LSc2ab, human passage (B341)	47×10^{3}	22×10^{2}	1.3	95.3

* PFU/0.2 ml.

† Control titer — titer in presence of dextran sulfate.

contrast the virus in one of the plaques(9) was much less sensitive than the original vaccine virus. The relative insensitivity of the virus in this plaque (PR_T 97.8) corresponded to the 20-fold reduction in sensitivity brought about by a single human passage of the original vaccine, as indicated by PR_T values for strain B341 of 94.9 (Table I) and 95.3 (Table II). These results emphasized that inhibition by dextran sulfate alone did not adequately identify LSc2ab virus.

Dextran sulfate and equine serum. Sixteen wild type 1 polioviruses were tested for sensitivity to the combined LSc2ab inhibitors. As shown in Table III, all 16 strains were largely unaffected by concentrations of equine serum (H_3) and dextran sulfate that strongly inhibited the LSc2ab vaccine. There was a slight reduction of the plaque number of most of the strains (mean PR_T of 23), and a similar reduction also occurred consistently on testing of the Mahoney and CHAT strains (PR_T of 33 and 23, respectively). In addition there was regularly a slight reduction in plaque diameter (mean PR_D of 30), but these minor effects did not interfere with recognition of plaques. Strain 143 was a partial exception in that it was more strongly inhibited than the other wild strains, as reflected in the reduction both in number (PR_T 80) and size (PR_D 70) of plaques. Yet even this strain was easily distinguishable from LSc2ab (PR_T >99.9 and PR_D 100) or its fecal passages. The possibility that there was a minor subpopulation of sensitive virus, detectable by clonal analysis of strain 143, was not examined.

Plaque formation of 4 unpassed fecal isolates of LSc2ab was completely suppressed by the combination of inhibitors (Table IV). Since the titers of the unpassed fecal viruses were not very high, the full extent of inhibition could not be determined, but the titers were reduced by at least $10^{2.3}$, $10^{1.9}$ and $10^{1.8}$ and $10^{3.2}$ PFU/ml for LSc2ab strains B407, B411 and B413, and B341, respectively. Moreover, no plaques at all appeared even after 4 days in the presence of the inhibitors. In contrast dextran sulfate alone had substantially less effect as noted earlier (Table I).

Discussion. Although strain-specific antisera to the LSc2ab virus permit identification of the LSc2ab vaccine, their value is sharply limited in the situation where they are most needed, namely, for discrimination among homotypic polioviruses isolated from the feces. This is a consequence of a loss of immunologic specificity resulting from antigenic changes that occur in polioviruses during human passage. The frequency of such changes is directly related to the duration of multiplication of virus in the intestine(3,6,7).

TABLE III. Differentiation of LSc2ab Virus from Wild Type 1 Strains.

Virus strain	Control (2.5% FCS)	Equine serum (H_3, 1.5%) + dextran sulfate (100 μg/ml)	Reduction in plaque size (%) PR_T	PR_D
2	7.1*	6.9*	33	44
5	7.2	6.9	53	31
7	7.0	7.0	0	17
8	7.0	6.9	11	47
9	7.3	7.3	2	12
12	7.0	6.9	11	36
20	7.3	7.2	19	38
32	7.3	7.3	6	50
36	7.3	7.1	39	44
51	7.2	7.0	39	25
61	7.4	7.4	4	21
78	7.3	7.2	22	11
86	7.2	7.3	−9	11
118	6.2	6.0	35	57
121	7.3	7.3	−2	7
143	5.7	5.0	80	70
Controls:				
LSc2ab	6.8	<4.0	>99.9	100†
CHAT	7.3	7.2	23	17
Mahoney	7.9	7.8	33	40

* Log_{10} PFU/ml.

† PR_D on monolayers infected with 10- and 100-fold greater concentrations of LSc2ab remained >90; only pinpoint plaques (<0.5 mm) were observed.

Normal bovine seras have been found that are quite specific in their ability strongly to inhibit plaque formation of the CHAT type 1 poliovirus(8). Sensitivity to such sera is genetically determined(8,9). However, neither bovine nor equine sera that selectively inhibit the LSc2ab vaccine alone have been found. As an alternative an equine serum that strongly inhibited plaque formation of the LSc2ab virus while only weakly inhibiting the CHAT and Mahoney viruses was selected. By fractionation the serum was rid of its anti-CHAT activity so that the inhibition produced by the serum affected only the LSc2ab strain (1). This was possible since the anti-CHAT activity was contained in the 19S globulin-containing fractions, whereas the anti-LSc2ab activity was associated with the 7S globulins. The 7S fractions of this equine serum gave a striking plaque reduction of LSc2ab and had no effect whatever on the CHAT strain(1). A similar result was obtained by treatment of the equine serum with 2-mercaptoethanol (Pagano, J. S., unpublished). The fractionated equine serum free of 19S globulins was of value for quantitatively distinguishing individual strains in double infections with the CHAT and the LSc2ab type 1 strains(10). However, since the anti-Mahoney activity was also associated with the 7S globulins, the fractionated serum did not serve to distinguish LSc2ab virus from this representative virulent type 1 strain (Mahoney).

Dextran sulfate not only inhibits plaque formation of the LSc2ab strain (m^-), but also enhances plaque formation of the CHAT, Mahoney and wild type 1 strains (m^+)(3,5). The m^+ character of the latter group of viruses persists after human passage in the case of the CHAT strain(3). The use of dextran sulfate together with inhibitory equine serum not only reinforced the inhibition of LSc2ab, but the substance also prevented a significant inhibition of Mahoney and CHAT virus by the equine serum(2). The same action, namely, lack of inhibition, was predicted for other m^+ strains, in particular wild type 1 viruses. The use of both inhibitors in combination to distinguish wild strains was expected to be superior to the use of dextran sulfate alone, since the rather frequent m^+ mutants in m^- strains probably would remain ho^-. This is readily tested since m^+ mutants are easily isolated from LSc2ab virus. The converse, do ho^+ (nonsensitive) mutants remain m^-, can be tested by deliberate induction and propagation of ho^+ mutants(11).

In any case the likelihood of double mutations arising in LSc2ab virus during intestinal passage that would render it nonsensitive to both inhibitors seems exceedingly small. However, for final proof this requires further

TABLE IV. Identification of LSc2ab Virus After Human Passage.

LSc2ab fecal isolate*	Control (2.5% FCS)	Equine serum (H_3, 1.5%) + dextran sulfate (100 μg/ml)
B407 (39 days)	3.3†	<1.0†
B411 (25 ")	2.9	<1.0
B413 (25 ")	2.9	<1.0
B341 (8 ")	4.4	1.2
LSc2ab vaccine (control)	6.9	<4.0

* 10% fecal suspensions tested directly without cell passage in plaque reduction tests. Day of collection of stool after administration of vaccine in parentheses.

† Log_{10} PFU/ml.

testing of a larger number of isolates of LSc2ab after human passage, especially virus isolated 3 and 4 weeks after administration of the LSc2ab vaccine. The 4 fecal strains of LSc2ab available for testing were all inhibited by the 2 markers, but isolates containing high titers of virus would be needed to determine the limits of the inhibitory effect.

It seems reasonable to conclude even after testing only 17 strains that wild strains sensitive to this combination of inhibitors are rare. This tentative conclusion gains support from the unselected nature of the wild strains tested — all except one only slightly if at all effected by the inhibitors — and from published information which indicates that most strains of wild type 1 virus are not inhibited by even one of the inhibitors used alone(5,11). The one wild strain that was partially inhibited by the equine serum and dextran sulfate was still distinguishable from LSc2ab. In such cases graded concentrations of equine serum and dextran sulfate could be tried since the range of concentrations of serum inhibited LSc2ab is large, whereas the range affecting even partly sensitive wild virus is apt to be much narrower.

Summary. The antiviral properties of two substances, dextran sulfate and equine serum, were made use of in combination to distinguish the LSc2ab (Sabin) strain of type 1 poliovirus from other type 1 strains. The combination of inhibitors strongly suppressed plaque formation of LSc2ab virus. In plaque reduction tests with the combined inhibitors 17 or 18 strains of type 1 poliovirus, including attenuated, wild and virulent strains, were unaffected, and these were readily distinguished from the LSc2ab virus.

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Selective Inhibition of Deoxyribonucleic Acid Synthesis by Salicylhydroxamic Acid.* (31369)

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Previous communications regarding hydroxyurea and other hydroxamic acids have described various pharmacological actions of compounds of this type(1-7); consequently a number of other hydroxamic acids are being synthesized and investigated regarding antimicrobial and antitumor properties, effects on the central nervous system, and mode of metabolic alteration.

In the course of testing several such compounds to determine effects on deoxyribonucleic acid synthesis *in vitro,* salicylhydroxamic acid was noted to exert a selective effect on the test system; a report follows.

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