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Intestinal Trypsin Can Significantly Modify Antigenic Properties of Polioviruses: Implications for the Use of Inactivated Poliovirus Vaccine

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It was recently reported that the intestinal protease trypsin cleaves in vitro the VP1 protein of type 3 poliovirus at antigenic site 1 (J. P. Icenogle, P. D. Minor, M. Ferguson, and J. M. Hogle, J. Virol. 60:297–301, 1986). We found that incubation of purified or crude type 3 poliovirus preparations with specimens of human intestinal fluid brings about a similar change in the virion structure. Sera from children immunized solely with the regular inactivated poliovirus vaccine (IPV) neutralized trypsin-cleaved Sabin 3 virus poorly, if at all, despite moderate levels of antibodies to the corresponding intact virus. Sera containing very high titers of the intact virus also neutralized the trypsin-cleaved virus but at a relatively weaker capacity. Most sera from older persons who may have been exposed to a natural poliovirus infection before the introduction of the poliovirus vaccines as well as sera from children infected with type 3 poliovirus during the recent outbreak in Finland were able to neutralize the trypsin-cleaved type 3 polioviruses. Serum specimens collected 1 month after a single dose of live poliovirus vaccine from children previously immunized with IPV were able to neutralize the trypsin-cleaved virus as well. During natural infection and after live poliovirus vaccine administration polioviruses are exposed to proteolytic enzymes in the gut. Our results may offer an alternative explanation for the relatively weak mucosal immunity obtained with IPV. Improvement of IPV preparations by incorporation of trypsin-treated type 3 polioviruses in the vaccine should be studied.

Three antigenic sites involved in virus neutralization have been identified in polioviruses (10) and located at the surface of the poliovirion (4). Antigenic site 1 composed of amino acids 89 to 100 of VP1 (virion protein 1) is a major immunogenic site for type 2 and 3 polioviruses, as judged by monoclonal antibodies induced in mice (10). Relative importance of the three sites in human immunity is not known. It was reported recently that trypsin, a pancreatic serine protease present in the intestinal fluids, can cleave both type 1 and 3 polioviruses (Sabin strains) at antigenic site 1 (2, 7). While the virus in both cases retains its infectivity, the antigenic properties of type 3 poliovirus are drastically altered, and trypsin-cleaved viruses are not neutralized or immunoprecipitated by site 1-specific monoclonal antibodies (7).

Oropharyngeal mucosa is considered to be the primary site of poliovirus replication in humans. The infection is subsequently spread both through viremia and directly via the gastrointestinal tract to the intestines, where the replication can continue for several weeks despite neutralizing antibodies in the circulation. Since the progeny in the intestinal lumen are exposed to trypsin and other proteolytic enzymes they may also escape from neutralization in vivo by site 1-specific antibodies induced by intact virus (11). During a recent outbreak of poliomyelitis in Finland (5), an antigenic drift of the causative type 3 poliovirus strain was found to have taken place in several infected persons (6), with trypsin-sensitive variants being selected against a trypsin-resistant parental strain (6, 11). The host enzyme-mediated proteolytic modification of antigenic properties may thus allow the virus to extend its replication in a given host. Eventually, the host immune system will respond to the cleaved viruses as well, and the infection is eliminated.

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Immunization with the inactivated poliovirus vaccine (IPV) was originally reported to result in a relatively weak mucosal immunity as compared with those obtained through natural infection or with the oral (live) poliovirus vaccine (OPV). This has been associated with differences in the vaccine-induced mucosal virus-specific immunoglobulin A responses (12). More recently it has been reported that at least some IPV preparations are capable of inducing mucosal poliovirus-specific immunoglobulin A secretion (1). There is also direct evidence that secretion of mucosal immunoglobulin A may be needed for efficient elimination of intestinal poliovirus infection (E. Savilahti, T. Klemola, B. Carlsson, L. Mellander, M. Stenvik, and T. Hovi, J. Clin. Immunol., in press).

As antigenic site 1 in IPV remains uncleaved during immunization, we wanted to examine whether antibodies induced by IPV are capable of neutralizing trypsin-cleaved type 3 polioviruses. We also studied the effects of a single OPV dose and that of a natural, wild-type type 3 poliovirus infection on the subsequent capacity of serum antibodies to neutralize trypsin-cleaved type 3 polioviruses.

MATERIALS AND METHODS

Serum specimens. Three sets of serum specimens were studied. A set of 54 specimens representing different age groups was selected from the routine diagnostic material of the Department of Virology, University of Helsinki, in 1977 and stored frozen at −20°C since then. Twenty-two sera drawn from 1-year-old infants 1 month after the second dose of a high-antigen-content experimental IPV (32 D-antigen units of Formalin-inactivated type 3/Saukett virus per dose) were kindly made available by K. Lapinleimu and M. Stenvik of this Institute. The third set of sera comprised pre- and postvaccination specimens from a group of 17 6-year-old children examined for wild-type type 3 poliovirus excretion

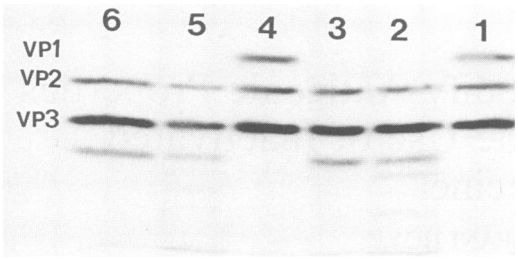

FIG. 1. Cleavage of VP1 in purified and crude type 3 poliovirus (Sabin) preparations by trypsin and human intestinal fluid. Purified [^{35}S]methionine-labeled poliovirus (type 3/Sabin) was treated with trypsin or human intestinal fluid as described in Materials and Methods and analyzed for protein composition by polyacrylamide gel electrophoresis (denaturing conditions, 10 to 20% gradient gel discontinuous buffer system of Laemmli [8]) followed by an electrophoretic transfer to a nitrocellulose filter and autoradiography. Purified virus was analyzed as such (lane 1) or after treatment with trypsin (lane 2) or intestinal fluid (lane 3). A sample of ^{35}S-labeled purified virus was mixed with crude virus preparation and analyzed as such (lane 4) or after digestion with trypsin (lane 5) or intestinal fluid (lane 6).

during the recent outbreak of poliomyelitis in Finland (5) and also studied for vaccine virus excretion and antibody responses during the subsequent nation-wide OPV campaign (13).

Virus preparations. Type 3 poliovirus, strain Sabin, used in the OPV, was propagated in Vero cells and purified by sucrose gradient centrifugations (14). Trypsin-cleaved virus was prepared as described by Icenogle et al. (7) using purified type 3/Sabin virus. Trypsin treatment of crude type 3/Sabin virus resulted in an antigenic change similar to that of purified virus (see below). Thus, for the following antibody assays crude virus preparations were incubated overnight at 4°C with 80 μg of purified trypsin per ml. The digestion was stopped by adding fetal calf serum up to 5%. Digestion with specimens of human intestinal fluid (kindly provided by E. Savilahti, Children's Hospital, University of Helsinki) was done in a similar way.

Antibody assays. Neutralizing antibodies in the sera were measured either by using endpoint titration and 50 to 100 50% tissue culture infective dose units of intact virus in Vero cells (13) or by determining the neutralization index with regard to intact and trypsin-treated virus preparations. For the latter, 10 μl of undiluted test serum was incubated with 30 μl of virus preparation (about 10^6 PFU/ml) for 1 h at 37°C, and the titer of remaining infectious virus was determined in a plaque assay in HeLa cells. The logarithm of the ratio of the titer of the respective virus preparation incubated without serum to that obtained with the test serum is used as the numerical value of the neutralization index of the test serum. On the basis of repeated tests with standard virus and serum preparations, a reduction of the virus titer by at least 35% (0.5 log) was required to conclude that the serum had neutralizing capacity.

Analysis of antigenic site 1. Integrity of antigenic site 1 in virus preparations was examined by using a plaque neutralization assay as above, with two site 1-specific and three other type 3-specific neutralizing monoclonal antibodies (7). The monoclonal antibodies were a generous gift from Morag Ferguson, London.

Statistical analysis. Since the results of antibody assays did not show normal distribution we used exclusively nonparametric statistical methods in the evaluation (3). Results on groups of persons with different immunization histories or ages were compared by using the Mann-Whitney test with continuity correction. Within groups, the results on assays with the two virus preparations were analyzed for dissimilarity by using the Wilcoxon signed rank test and examined for positive correlation by the Spearman method. The Spearman method was also used for examining correlation between age and antibody levels. The Wilcoxon signed rank test was also used to evaluate individual antibody increases.

RESULTS

Cleavage of VP1 at antigenic site 1 in crude poliovirus preparations by trypsin and human intestinal fluid. Others have previously shown that purified type 3 poliovirus is cleaved by trypsin at antigenic site 1 (2, 7). We found that a similar cleavage takes place in crude virus preparations (Fig. 1) and results in a similar antigenic change as documented by a selective resistance to neutralizing monoclonal antibodies specific for intact site 1 (Table 1). Furthermore, incubation of purified or crude type 3 poliovirus preparations with specimens of human intestinal fluid brought about similar biochemical (Fig. 1) and immunological (Table 1) results. Practically identical results were obtained with intestinal fluid specimens from two individuals.

Age-dependent prevalence of neutralizing antibodies to trypsin-cleaved type 3 polioviruses. Sera collected from different age groups in 1977, more than 10 years after the "elimination" of poliomyelitis in Finland (9), were tested for neutralizing antibodies to trypsin-treated and intact poliovirus type 3. Since widespread circulation of polioviruses in the population had been stopped rather abruptly at about 1960 (9), persons younger than 17 years in 1977 most probably had neutralizing antibodies in their sera solely as a result of immunization with IPV. Older persons, in contrast, had, in principle, had an opportunity to be exposed to wild polioviruses. Most sera collected from children 16 years of age or younger were unable to neutralize trypsin-cleaved virus, while most sera from the older age group neutralized it (Fig. 2). The difference between the two age groups was statistically (Mann-Whitney) significant ($P < 0.001$). Within the age group older than 20 years, the neutralization index did not show a correlation with the age ($P > 0.2$). However, sera from the older age group had a better capacity to

TABLE 1. Antigenic analysis of purified and crude type 3 poliovirus (Sabin) after incubation with trypsin or human intestinal fluid

Virus	Neutralization		
	Mc 175[a]	Mc 882[b]	Mc 879[c]
Purified virus			
Control	+	+	+
Trypsin treated[d]	−	+	+
Intestinal fluid treated[e]	−	+	+
Crude virus			
Control	+	+	+
Trypsin treated	−	+	+
Intestinal fluid treated	−	+	+

[a] A site 1-specific monoclonal antibody. Similar results in all cases were obtained with another site 1-specific monoclonal antibody (Mc 204).
[b] A site 2-specific monoclonal antibody.
[c] A site 3-specific monoclonal antibody.
[d] Trypsin treatment for 24 h at 4°C with 1 μg of trypsin per μg of viral protein (7) for the purified virus and 80 μg of trypsin per ml for the crude virus preparation.
[e] Intestinal fluid was used undiluted.

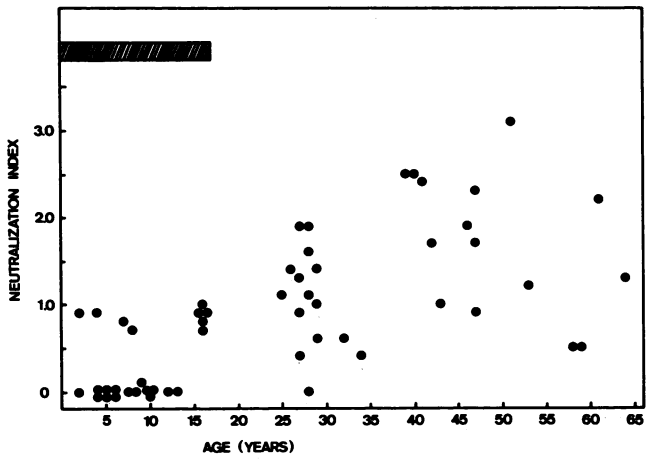

FIG. 2. Neutralization of trypsin-treated type 3 poliovirus (Sabin strain) by sera collected from different age groups in 1977 in Finland. The horizontal bar indicates the age groups which most likely have not been exposed to circulating wild poliovirus and have been immunized solely with the IPV.

neutralize ($P < 0.001$) the intact virus as well (Table 2), and the ratio of intact virus antibodies to cleaved virus antibodies was similar in both groups (Fig. 3; Table 2).

Antibodies to trypsin-cleaved poliovirus induced by an experimental high-antigen-content IPV preparation. Since the IPV preparation previously used in regular immunization in Finland is considered to be relatively weakly immunogenic (5, 9) and since the above results could also be interpreted to suggest that the relative immunogenicity rather than the nature of the primary immunogen is important, we wanted to study the neutralization capacity of antibodies induced by a more immunogenic IPV preparation.

Sera collected 1 month after the second dose of an experimental high-antigen-content vaccine had without exception a very good ability to neutralize the intact virus. Most of the sera also readily neutralized the trypsin-cleaved virus. The neutralization indices for the latter virus were, however, definitely ($P < 0.001$) lower (Table 2). Individual sera showed large variations in the relative capacity to neutralize the cleaved virus as compared with that for the intact virus, while as a group the ratio of the neutralization indices against the intact virus versus trypsin-cleaved virus was similar to that found above in the 1977 sera (Table 2). Neutralization indices against the trypsin-cleaved virus showed a good correlation ($P < 0.001$) (Spearman test) with

TABLE 2. Neutralizing antibodies to intact and trypsin-cleaved type 3 poliovirus (Sabin strain) in sera from different age groups from 1977 and from vaccinees in a trial[a]

Age group (*n*)	Neutralization index			
	Median		Geometric mean	
	Intact	Cleaved	Intact	Cleaved
<20 years (24)	0.9	0	1.0	0.1
20–65 years (30)	2.3	1.3	2.2	1.4
Infants[b] (22)	3.2	2.2	3.1	2.1

[a] The correlation of neutralization index against the trypsin-cleaved virus versus that against the intact virus in individual sera (Spearman rank correlation) was significant ($P < 0.001$) in both age groups of the 1977 sera.

[b] Sera drawn from children immunized with an experimental vaccine (see text).

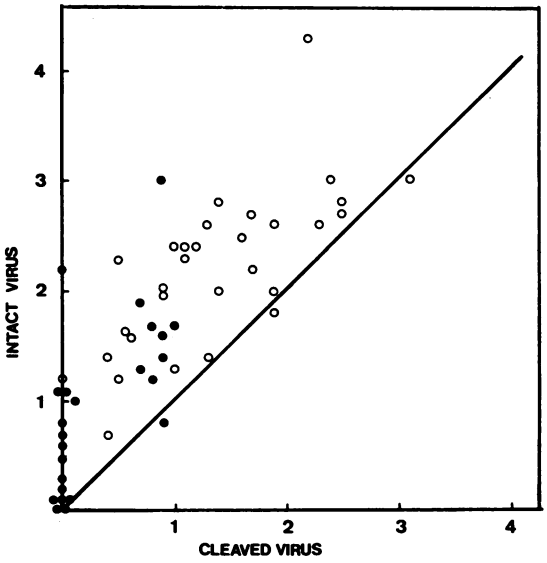

FIG. 3. Neutralization indices to intact and trypsin-cleaved type 3 poliovirus in sera collected in 1977 from persons younger than (●) or older than (○) 20 years of age. The Spearman rank correlation coefficient was 0.714 for the younger age group and 0.772 for the older age group ($P < 0.001$ in both cases).

the neutralization titer against the intact virus (Fig. 4). Trypsinized virus could not be used in the endpoint titer determinations because the test requires several replication cycles during which an intact virus is generated.

Appearance of antibodies to trypsinized type 3 poliovirus in serum after poliovirus infection or OPV vaccination. To study the effect of documented natural infection and that of OPV on the antibody patterns, we analyzed a further set of serum

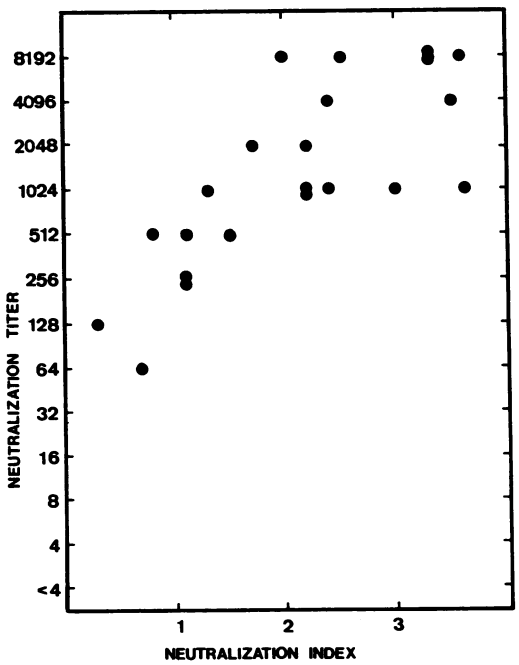

FIG. 4. Correlation between neutralization titers against intact type 3/Sabin virus and neutralization indices against trypsin-cleaved virus in sera from children immunized with the experimental IPV.

specimens. A group of 17 children had been examined in November 1984 for virus excretion because they were classmates to a boy with poliovirus-induced aseptic meningitis (5). Apart from this boy, five healthy children were also found to excrete the epidemic P3/FINLAND/84 virus (13). All the children had been previously vaccinated according to the national schedule, i.e., with three to four doses of the regular IPV. In November 1984 they were given an extra dose of IPV, and in early March 1985, along with the nation-wide OPV campaign, they were given a dose of trivalent OPV.

Serum specimens collected before and 1 month after the OPV dose were examined for neutralizing antibodies to intact and trypsin-cleaved poliovirus type 3. Pre-OPV sera from the six children who had been excreting the epidemic virus 4 months earlier neutralized the trypsinized Sabin 3 virus as well as the intact virus (probability for a mistake when assuming dissimilarity is greater than 0.062). In contrast, the neutralizing capacity of sera from the assumed nonexcretors was weaker ($P < 0.018$) when tested against the trypsinized virus as compared with that against the intact virus (Fig. 5). The level of pre-OPV antibodies to the cleaved virus was higher ($0.025 < P < 0.05$) in the known P3/FINLAND/84 excretors than in the assumed nonexcretors. The levels against the intact virus were, in contrast, similar ($P > 0.2$) in the two groups of children.

OPV brought about a serological response to both the intact and the trypsinized Sabin 3 virus in the assumed nonexcretors ($P = 0.018$) but not in the known excretors (Wilcoxon signed rank test, $P > 0.062$).

Antibody levels before OPV to the trypsin-cleaved virus correlated well with those against the intact virus both in the known P3/FINLAND/84 excretors ($r_s = 0.90$, $0.02 < P < 0.05$) and in the assumed nonexcretors ($r_s = 0.74$, $0.02 < P < 0.05$). After OPV, however, no statistically significant correlation was found in either group ($P > 0.2$ and $P > 0.5$, respectively).

DISCUSSION

Our results suggest that the epitope distribution of neutralizing type 3 poliovirus antibodies induced by IPV is different from that brought about by natural infection or OPV. We also showed that incubation of poliovirus type 3/Sabin with specimens of normal intestinal excretions can modify the antigenic properties of the virus in a way similar to that previously reported for trypsin and shown to be due to the cleavage of antigenic site 1 in VP1 (2, 7). The cleavage of VP1 and the concomitant antigenic change at site 1 brought about by intestinal fluid can be specifically prevented by inhibitors of trypsin, suggesting that trypsin in the intestinal fluid is responsible for these changes (M. Roivainen, A. Huovilainen, and T. Hovi, manuscript in preparation). Type 3/Sabin and trypsin-sensitive wild strains in fecal extracts share the altered antigenic properties of site 1 with trypsin-cleaved viruses but rapidly achieve normal reactivity with site 1-specific monoclonal antibodies during growth in cell culture (11). Consequently, it is pertinent to assume that during natural poliovirus infection and as a result of OPV immunization, antibodies will be formed to the protease-cleaved poliovirus as well. In the mouse, trypsin treatment of the type 3/Sabin virus appears to improve the immunogenicity of antigenic sites 2 and 3 (7, 11). Our present results (Fig. 5) suggest that the relative share of non-site 1 antibodies is increased after natural infection and after OPV as compared with that induced by IPV. During IPV immunization, site 1 most likely remains intact and the resulting antibodies are targeted to uncleaved site 1 and to a lesser extent to other antigenic sites in the virion. While we believe that the enhanced immunogenicity of the other antigenic sites after cleavage of site 1 is the main reason for the above observations, we cannot formally exclude other alternatives, such as modification of the antigenic sites during the IPV inactivation or different antigen presentation in the gut versus that after a subcutaneous injection as putative contributing factors. A further possibility could be that the Saukett and Sabin 3 strains used in the IPV and OPV, respectively, significantly differ from each other at antigenic sites other than site 1. Results similar to those described above for the type 3/Sabin strain were, however, obtained in a selected serum material tested with trypsin-treated Saukett virus (unpublished data).

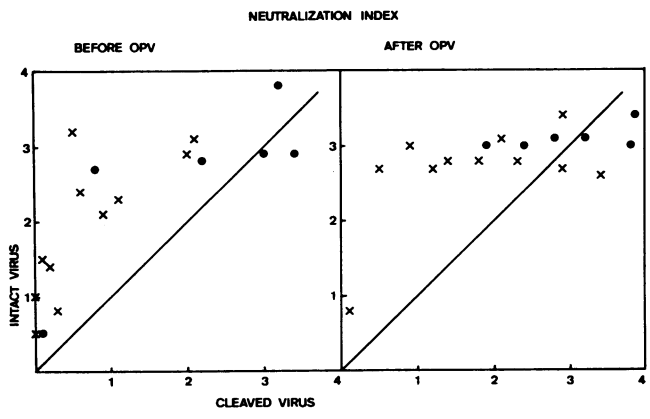

FIG. 5. Pre (left panel)- and post (right panel)-OPV neutralization indices to intact and cleaved type 3/Sabin virus in children exposed to wild-type type 3 poliovirus 4 months earlier with resulting virus excretion (●) or without evidence for virus excretion (assumed nonexcretors) (×).

In the present material the median of IPV-induced neutralization indices to the trypsin-cleaved virus was about one unit lower than that to intact virus. This indicates that the median capacity of the tested post-IPV sera to neutralize the trypsin-cleaved virus was only about 10% of that against the intact virus. This difference between IPV and OPV may contribute to the observed relative weakness of the mucosal immunity achieved with the early preparations of IPV (12).

On the basis of the uneven distribution of monoclonal antibodies between the three known antigenic sites of poliovirus (10), site 1 has been considered to be the most immunodominant one in the mouse, as regards the type 3/Leon or Sabin strains. Our results show that it is also of major importance in humans when immunization is done by using intact viruses such as the type 3/Saukett in the IPV. This conclusion is further supported by the observation that during the recent outbreak resulting from type 3 poliovirus in Finland, new variants appeared to be selected on the basis of susceptibility to trypsin at site 1 (6, 11). On the other hand, the failure of antibodies induced by the regular IPV to provide protection against trypsin-treated poliovirus 3 and the rapid appearance of antibodies with this specificity after natural infection or OPV indicates that, in vivo, virion surface structures other than the intact site 1 are important in the induction of protecting antibodies to the cleaved type 3 poliovirus. Our data on the high-titered sera showed that these other sites can be immunogenic in IPV preparations as well. At the moment we do not know whether the antibodies neutralizing trypsinized type 3/Sabin are targeted to the

known sites 2 and 3 in the virion (10) or whether they recognize the trypsinized site 1 or an as yet unidentified structure in the virion.

Our results with the high-titered sera from children immunized with the experimental high-antigen-content IPV demonstrated a good general correlation between the endpoint neutralization titer against the intact virus and the neutralization index to the trypsin-cleaved virus, as well as between the neutralization indices against the two virus preparations. However, individual sera showing a very low or nonmeasurable level of neutralizing antibodies to trypsin-cleaved type 3 poliovirus were also found in these subjects. Furthermore, for practical purposes it has to be remembered that the endpoint titers measured in these sera against intact type 3/Sabin virus are exceptionally high. The sera were from a vaccine trial utilizing higher antigen doses than used in the regular vaccinations in any country at the moment. Most sera from persons immunized with the regular IPV only did not significantly neutralize trypsin-cleaved type 3 poliovirus. Further increase of the antigen content of the regular vaccines would make the vaccines even more expensive than at present. An IPV preparation supplemented with trypsin-cleaved type 3 poliovirus might be a more practical approach and should be properly tested.

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