Prior Immunity to Homologous and Heterologous Salmonella Serotypes Suppresses Local and Systemic Anti-Fragment C Antibody Responses and Protection from Tetanus Toxin in Mice Immunized with Salmonella Strains Expressing Fragment C

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We have investigated the effect of preexisting immunity to homologous (Salmonella typhimurium) or heterologous (S. dublin) serotypes of Salmonella on the ability of an attenuated S. typhimurium arsA aroD vector (BRD509) to immunize mice against the heterologous antigen fragment C (FrgC). We studied two strains, BRD847 and BRD937, expressing FrgC carried on plasmids that differ only with respect to the promoter controlling FrgC expression, the nirB promoter in the case of BRD847 and the htrA promoter in the case of BRD937. Mice were preimmunized orally with S. typhimurium BRD509, S. dublin aroD (BRD620), or saline. Forty-four days later, they were immunized orally with BRD847 or BRD937. Prior immunity to S. typhimurium severely depressed the serum immunoglobulin G (IgG) and IgA anti-FrgC response in both BRD847- and BRD937-immunized mice. Mice with existing immunity to S. dublin also had lower IgG anti-FrgC geometric mean titers (GMTs) than did mice preimmunized with saline, but this difference was significant only in the case of mice immunized with BRD937. However, in nonimmune mice or in mice preimmunized with S. typhimurium or S. dublin, the anti-FrgC IgG GMTs were always higher in mice in the BRD937 groups than in the equivalent BRD847 groups. This is reflected in the effect of prior immunity on the ability of oral immunization with BRD847 or BRD937 to protect mice from challenge with a lethal dose of tetanus toxin. All of the mice preimmunized with saline and then immunized with BRD847 or BRD937 survived challenge. Only 20% of the animals immunized with BRD847 and 60% of the mice in the BRD937 group survived tetanus toxin challenge if they were preimmunized with BRD509. Preexisting immunity to S. dublin did not affect the ability of BRD937 to immunize mice against tetanus, but it did reduce the efficiency of BRD847: only 60% percent of the mice survived challenge. The intestinal secretory IgA responses to FrgC were very similar in the BRD847 and BRD937 groups. Prior immunity did depress the IgA anti-FrgC titers but only significantly so in the mice preimmunized with BRD509. These results show that preexisting Salmonella immunity, particularly to homologous serotypes, can severely compromise the ability of live Salmonella vectors to deliver heterologous antigens to the mammalian immune system. However, the results also indicate that this may be overcome by the design of more powerful in vivo expression systems.

Defined nonreverting attenuated strains of Salmonella are being investigated as live vaccines against salmonellosis and as live vectors for delivering heterologous antigens to immune systems of humans and animals (3, 14, 21). Mutations in a number of different genes have been shown to attenuate Salmonella spp. and render them useful as live vaccine and vector strains (3, 14, 21).

A variety of antigens from a number of different organisms have been expressed in Salmonella carriers and have been used to immunize humans and animals (3, 14, 21). Immunization with recombinant Salmonella strains can induce antibody (secretory and circulating) and cellular response to the heterologous antigen. Live Salmonella vaccines can be administered orally, avoiding the need for injections and the possible risk of blood-borne infections that can arise from the reuse of needles, a practice common in the developing world.

Great variation in the immune response to different foreign antigen expressed in Salmonella vectors has been reported (3, 14, 21). A number of parameters that might influence the response to the foreign antigen have been examined; these include expression levels, constitutive versus inducible expression, carrier strain, cellular location of the antigen, and route and number of immunizations (21). One factor that might have a great effect on the efficacy of the Salmonella carriers, the existence of preexisting immunity to the carrier strain, has not been investigated in depth. The few studies performed to date have produced conflicting results; two studies showed prior immunity to the salmonella vector enhanced the response to the foreign antigen, and a third demonstrated a suppressive effect on the response to the heterologous antigen (1, 2, 29).

Prior immunity is known to greatly reduce the effectiveness of live viral vectors (23, 30). We have been developing a single-dose oral tetanus vaccine by using fragment C (FrgC)-expressing Salmonella (6, 8, 12, 21, 22). There is a great need for such a vaccine in the developed
world, where nearly 1 million people die from tetanus every year (26). The disadvantages of using the current tetanus vaccine (tetanus toxoid) in the developing world are the need for a cold chain, the need to administer the vaccine by injection, and the need for multiple immunizations.

We have a great deal of experience with expressing FrgC in Salmonella and of the effects that of variables such as gene copy number and the type of promoter can have on the subsequent immune response following immunization (21). We can now consistently induce 100% protection from tetanus in mice by immunizing with a single oral dose of an attenuated Salmonella strain carrying a plasmid which encodes the FrgC gene under the control of the nirB or htrA promoter (6, 22).

In this study, we determined the effect of prior immunization with the carrier strain alone (Salmonella typhimurium aroA aroD) or with a heterologous Salmonella serotype, S. dublin aroA aroD, on the circulating and secretory antibody response to FrgC and protection from tetanus in mice immunized with S. typhimurium aroA aroD expressing FrgC. The effect on the anti-FrgC response of expressing FrgC from different promoters in the face of prior immunity was also examined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. S. typhimurium BRD509 (SL1344 aroA aroD), BRD847 (BRD509/pTETnir15), and BRD937 (BRD509/pTEThtrA1) and plasmids pTETnir15 and pTEThtrA1 have been described previously (6, 22). The S. dublin aroA aroD strain (BRD620) was a gift from Cliff Hayward. Bacteria were routinely cultured aerobically in L broth or on L agar (9). Ampicillin was included in the growth medium used for BRD847 and BRD937.

Immunization. For immunization of mice, salmonellae were grown statically overnight in L broth, recovered by centrifugation, and resuspended in sterile phosphate-buffered saline (PBS; pH 7.2) to approximately 1 × 1010 to 5 × 1010 CFU/ml. Female BALB/c mice (6 to 8 weeks old; Charles River, Margate, United Kingdom) were orally immunized with salmonella suspension (0.2 ml) administered by gavage tube as described previously (9). Viable counts were performed on all vaccines.

Measurement of the systemic and local anti-FrgC antibody response. Anti-FrgC-specific serum immunoglobulin G (IgG), IgA, and IgM antibody titers were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (20). Briefly, 96-well enzyme immunosassay/radio immunosassay plates (Costar, High Wycombe, United Kingdom) were coated with recombinant FrgC (50 μl; 2.5 μg/ml in PBS; overnight, 4°C), washed three times with PBS containing 0.05% (vol/vol) Tween 20 (PBST; Sigma), and then blocked with PBS containing 1% bovine serum albumin (BSA). After being washed, plates were incubated with serial dilutions of serum for 2 h at 37°C. All samples and reagents were diluted in PBST containing 0.1% BSA. Plates were washed, incubated with biotin-conjugated goat anti-mouse IgG, IgA, or IgM as appropriate (Sigma), and washed again. Then horseradish peroxidase-conjugated streptavidin (Dako, High Wycombe, United Kingdom) was added, and bound antibodies were visualized by adding o-phenylenediamine substrate (0.04% o-phenylenediamine in citrate-phosphate buffer [pH 5] containing 0.01% H2O2). After color development, the reaction was stopped with 3 M H2SO4 and absorbance was read at 490 nm. Absorbance values were plotted against dilutions, and titers were determined as the reciprocal of the highest sample dilution giving an absorbance of 0.3 optical density unit.

To determine the intestinal antibody response, fresh fecal pellets (two to four per mouse) were collected into microcentrifuge tubes. One milliliter of a solution consisting of 1% (wt/vol) BSA (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma) in PBS was added to each tube. After being incubated overnight at 4°C, the tubes were vortexed to disrupt all solid material and then centrifuged at 16,000 × g for 5 min. The supernatant was recovered and stored at −20°C until analysis. The fecal extracts were assayed by ELISA for FrgC-specific IgA as described previously (22). The protocol for the ELISA was essentially as described above. To correct for variation in IgA present in each sample, the total IgA concentration of each fecal sample was determined by an IgA-specific capture ELISA as described previously (22). Final results were calculated by dividing the FrgC-specific IgA endpoint titer by the total IgA (micrograms) in the fecal sample (22).

Tetanus toxin challenge. Mice were challenged with 0.01 μg (50 × the 50% lethal dose [LD50]) of purified tetanus toxin as previously described, and fatalities were recorded for 4 days (6).

RESULTS

We examined the effect of prior immunity to Salmonella spp. on the efficacy of an attenuated S. typhimurium strain, BRD509, to deliver a foreign antigen, FrgC of tetanus toxin, to the murine immune system. BRD509 is an aroA aroD mutant of S. typhimurium. The systemic and local antibody response to FrgC and protection from tetanus toxin were analyzed in mice with and without preexisting Salmonella immunity. Although most Salmonella strains are closely related, immunity to Salmonella infection is largely serotype specific (15–18, 24). To analyze if any influence of preexisting Salmonella immunity on the immune response to a foreign antigen expressed by a Salmonella carrier is serotype specific or nonspecific, we preimmunized mice either with the S. typhimurium carrier strain alone (BRD509) or with BRD620, an S. dublin aroA aroD mutant. S. typhimurium and S. dublin belong to different serogroups, B (S. typhimurium) and D (S. dublin). The effects of different expression systems were also examined. Two derivatives of BRD509, BRD847, and BRD937 which express FrgC were used in this study (6, 22). Both strains possess a plasmid that encodes the FrgC gene. The two plasmids, pTETnir15 and pTEThtrA1, are identical except that the FrgC gene is controlled by different promoters (6, 22). The nirB promoter controls FrgC expression in strain BRD847 (BRD509/pTETnir15); in strain BRD937 (BRD509/pTEThtrA1), FrgC is expressed from the htrA promoter (6, 22). These promoters are induced by different environmental cues, the former by anaerobiosis and the latter by stress such as elevated temperature (6, 22). There is also evidence that both promoters are upregulated as bacteria enter cells (11). A single oral immunization of mice with either BRD847 or BRD937 induces high levels of serum and secretory antibodies to FrgC and complete and long-lasting immunity to tetanus toxin and S. typhimurium.

Groups of 10 mice were preimmunized orally with ~1010 CFU of either BRD509 or BRD620; control mice received saline. Forty-four days later, mice were separated into groups of five and immunized orally with ~1010 CFU of either BRD847 or BRD937.

Effect of prior Salmonella immunity on the serum antibody response to FrgC. Serum samples were taken 14, 28, and 42 days after immunization with BRD847 or BRD937. The titers of IgG, IgA, and IgM anti-FrgC antibodies in the sera from individual mice were determined by ELISA, and the results are shown in Fig. 1. The anti-FrgC response was examined at several time points to see if prior immunity affected the kinetics of the anti-FrgC response as well as the magnitude of the response.

At all time points, the IgG anti-FrgC antibodies were significantly reduced (P < 0.05) in the sera of mice immunized with BRD847 or BRD937 (hereafter referred to as BRD847 or BRD937 mice) if they were previously immunized with the carrier strain BRD509. For example, at the peak of the response (28 days), the geometric mean titers (GMTs) of anti-FrgC IgG in the sera from the control mice (the saline-BRD937 group) and the BRD509-BRD937 mice were 329,570 and 2,060, respectively. The difference in the mean anti-FrgC titers between mice preimmunized with the homologous carrier (BRD509) or saline was greater for the BRD937 group (30-260-fold) than for the BRD847 group (20-40-fold). However, in both the saline and BRD509 mice, the anti-FrgC IgG titers were higher in the sera of BRD937 mice than those of the mice in the equivalent BRD847 groups. The kinetics of the IgG anti-FrgC response induced by BRD847 was altered by preimmunization with BRD509 because the peak response in the BRD509-BRD847 mice occurred at 14 days rather than 28
days as for the saline-BRD847 mice. The pattern of the serum IgA response was also different in these mice.

Pre-immunization with BRD509 also reduced the anti-FrgC serum IgA and IgM titers, although the magnitude of the decrease was less than for the IgG titers. The effect was greatest for the IgA response. The reduction in the IgA anti-FrgC GMT was significant ($P < 0.05$) for both the BRD847 and BRD937 groups at all time points; however, the difference in IgM anti-FrgC GMTs was not significant for any of the time points for the BRD847 group and for only the 28- and 42-day sera for the BRD937 group.

Preimmunization with $S$. dublin BRD620 also negatively affected the serum antibody response to FrgC in mice immunized BRD847 and BRD937. However, the effect was much lower than that seen for mice immunized with the homologous serotype. For the BRD620-BRD937 mice, the IgG anti-FrgC GMTs were significantly lower at the 28- and 42-day time points than for the saline-BRD937 mice. In contrast, there was no significant difference in titers between the saline-BRD847 and BRD620-BRD847 groups at any time point. However, BRD620-BRD937 mice mounted a stronger anti-FrgC antibody response than BRD620-BRD847 mice, and although immunity to $S$. dublin reduced the anti-FrgC IgG GMT in the sera of BRD937 mice 4- to 10-fold compared to the saline-BRD937 group, the GMTs were still higher than those of the saline-BRD847 group. Preimmunization with BRD620 also altered the kinetics of the IgG anti-FrgC response.

Preexisting immunity to $S$. dublin significantly compromises the IgA anti-FrgC response in mice immunized with BRD847. Immunization with BRD620 did not significantly reduce the IgA anti-FrgC GMTs in BRD937 mice, but it did affect the development of the response such that the peak of the response was delayed until day 28 rather than day 14 as was seen in the saline-preimmunized group. The IgM anti-FrgC titers were elevated in the mice preimmunized with BRD620 in both the BRD847 and BRD937 groups, although except for the 28-day sera from the BRD620-BRD937 mice, this increase was slight and nonsignificant.

**Effect of prior immunity on the intestinal IgA anti-FrgC response.** The intestinal anti-FrgC IgA response was measured in fecal extracts. ELISAs were performed on extracts from fecal pellets obtained 28 and 42 days after the second immunization because previous experiments had demonstrated that was when the peak response occurred in mice immunized with BRD847 and BRD937 (22). In the present study the strongest response was seen in the 28-day postimmunization fecal extract samples; the results for these samples are shown in Fig. 2. Similar specific anti-FrgC IgA titers were seen in the mice preimmunized with saline and then BRD847 or BRD937. Preimmunization with both the homologous and the heterologous
carrier strain reduced the intestinal response to FrgC in mice subsequently immunized with BRD847 or BRD937. As with the serum responses, the lowest anti-FrgC titers were measured in the samples taken from mice preimmunized with the homologous carrier strain. For both the BRD847 and BRD937 groups, the titers anti-FrgC IgA antibodies measured in fecal extracts of mice preimmunized with BRD509, but not BRD620, were significantly lower (P < 0.05) than those of mice in the saline group. The fold decrease in the fecal anti-FrgC IgA titers of mice preimmunized with BRD509 or BRD620 was very similar in the BRD847 and BRD937 groups.

**Effect of prior immunity on protection from tetanus.** To evaluate if the reduction in the antibody response to FrgC seen in the mice preimmunized with the *Salmonella* carriers was significant with regard to protective immunity, we challenged mice with tetanus toxin 53-days after the second immunization and recorded deaths for 4 days; the results are shown in Table 1. Mice that did not have immunological experience of *S. typhimurium* prior to immunization with BRD847 or BRD937 were fully protected against tetanus, confirming the previous report (22). Preexisting immunity to *S. typhimurium* greatly reduced the ability of BRD847 and BRD937 to induce protection against tetanus challenge. The effect was greatest in the BRD847 group: only 20% of the mice preimmunized with BRD509 survived tetanus toxin challenge. The BRD509-BRD937 mice were not as affected: 60% of the animals in this group were immune to tetanus. This finding reflects the higher mean anti-FrgC titers in the sera of BRD937 immunized mice. Immunity to BRD620 did not affect the ability of BRD937 to induce protection against tetanus (at the challenge dose of tetanus toxin used). Immunity to BRD620 did, however, compromise the effectiveness of BRD847 at inducing protection to tetanus, as only 60% of the BRD620-BRD847 mice were protected from tetanus challenge.

**DISCUSSION**

Preexisting immunity to *S. typhimurium* had a major negative effect on the immune response to FrgC in mice immunized orally with an *S. typhimurium aroA aroD* vector expressing FrgC. This was reflected in reduced serum and fecal anti-FrgC

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<tr>
<th>Group</th>
<th>1st immunization</th>
<th>2nd immunization</th>
<th>Tetanus toxin challenge survivors (%)</th>
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<tr>
<td>Saline</td>
<td>BRD847</td>
<td>BRD847</td>
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<tr>
<td>BRD620</td>
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<td>BRD847</td>
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* Mice were immunized as described in the text; 53 days after the second immunization, mice were challenged with 50 LD₅₀ of tetanus toxin, and the fatalities were recorded for 4 days.
antibody titers and protection against tetanus toxin compared to mice with no preexisting *S. typhimurium* immunity. The kinetics of the serum anti-FrgC response was also altered in some cases. The magnitude of the anti-FrgC response in the mice with preexisting immunity to *S. typhimurium* was dependent on the promoter used by the immunizing strain to express FrgC. The serum anti-FrgC antibody response was much stronger in mice immunized with strain BRD937, in which FrgC expression is under the control of the htrA promoter, than in mice immunized with BRD847, which expresses FrgC from the nirB promoter. This was also the case in the control mice preimmunized with saline. This confirms the superior immunogenicity of BRD937 with regard to the anti-FrgC response as was seen in a previous study (22).

Protection from tetanus toxin is mediated by serum antibodies. It is our experience that BRD847 and BRD937 induce the highest anti-FrgC antibody responses of a number of *Salmonella*-FrgC constructs that we have investigated (6, 22). A single oral dose of either BRD847 or BRD937 will provide solid long-lasting protection against tetanus. However, in the face of preexisting *S. typhimurium* immunity, both BRD847 and BRD937 were seriously compromised in the ability to induce immunity to tetanus. As would be expected from the serum anti-FrgC titers, preimmunization with BRD509 had a less dramatic effect on immunity to tetanus in mice immunized with BRD937 than on mice immunized with BRD847.

Existing immunity to a heterologous *Salmonella* serotype, *S. dublin*, also depressed the anti-FrgC response in mice receiving BRD847 or BRD937, although the effect was less severe than that seen in mice immune to *S. typhimurium*. The mean serum IgG anti-FrgC titers in mice given BRD620 and then BRD937 were significantly lower than in mice receiving BRD937 alone. However, the level of serum anti-FrgC antibodies in all of the BRD620-BRD937 mice was still sufficient to protect them from tetanus. In contrast, although the mean IgG anti-FrgC titers were not significantly lower in BRD620-BRD847 mice group than in saline-BRD847 mice, not all mice in the former group had anti-FrgC antibodies sufficient to protect them from the effects of tetanus toxin.

Preexisting *Salmonella* immunity also impaired the development of a mucosal antibody response to FrgC. The mean fecal IgA anti-FrgC titers were lower in the *Salmonella*-preimmunized mice than in the controls. As with the serum response, mice preimmunized with the *S. typhimurium* strain had the lowest anti-FrgC titers.

Of the other studies that have investigated the effect of prior immunity on the efficacy of *Salmonella* vectors, our results are in agreement with one and conflict with the other two (1, 2, 29). In terms of experimental design and vectors used, our study is closest to that of Bao and Clements (2). They reported that preexisting immunity to either *S. typhimurium* or *S. dublin* (induced by immunization with an araA mutant of each strain) augmented the local and systemic antibody response to the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) in mice immunized with an *S. dublin* araA carrier expressing LTB (2). The conflicting findings of our study and that of Bao and Clements (2) is unlikely to be explained by differences in the *Salmonella* carrier strains because both studies used strains that were attenuated due to mutations in the prechorismate (aromatic) pathway. The slight differences between the two studies in the preimmunization and immunization protocols would seem to be too minor to account for the vast differences seen. More likely, the opposing findings of the two studies may be accounted for by differences in the properties and cellular locations of the heterologous antigens studied: FrgC in our study and LTB in the work of Bao and Clements (2).

LTB is a good mucosal immunogen, whereas FrgC is not (10, 19, 19a, 20, 28). The two antigens also differ with respect to dependence on the viability of the *Salmonella* carrier to induce an immune response. Cardenas et al. (4) reported that mice immunized orally on four occasions with heat-inactivated *S. dublin* expressing LTB developed serum and intestinal anti-LTB titers that were statistically indistinguishable from those in mice similarly immunized with the viable strain. In contrast, two oral or intravenous doses of heat-killed *S. typhimurium* expressing FrgC failed to immunize any mice against tetanus toxin challenge (the antibody response was not determined [12]).

The locations of FrgC and LTB within the *Salmonella* cell are different. FrgC resides within the cytoplasm, whereas LTB is secreted into the periplasm (13, 25). It is likely that LTB, but not FrgC, is able to leak from the *Salmonella* cell. LTB needs to escape from the periplasm of enterotoxigenic *E. coli* to exert its effect but lacks specialized machinery to do so (13). As LTB is a good mucosal immunogen, LTB that has escaped from the *Salmonella* cell could induce a local and systemic antibody response. If this hypothesis is correct, then it is not surprising that LTB responses still occur when mice are immunized with *S. dublin* expressing LTB even in the face of preexisting anti-*Salmonella* immunity. The enhanced immunogenicity of LTB in the preimmunized mice may be due to a bystander effect arising from the anamnestic immune response to the *Salmonella* carrier.

Whittle and Verma reported that intraperitoneal priming with a *S. dublin* araA strain enhanced the subsequent antibody response to a viral B-cell epitope when they were immunized intraperitoneally with the same *S. dublin* strain expressing the B-cell epitope genetically fused to flagellin (29). It is not known if the route of immunization or the fact that only a B-cell epitope was expressed are important to the outcome of the study.

Our results are in agreement with those of Attridge et al. (1), who found that immunization with *S. Stanley* significantly compromised the serum antibody response to K88 in mice subsequently immunized with *S. Stanley* expressing K88. They found that the effect was less severe if the same preimmunizing but a different K88-expressing strain (*S. strasbourg*) was used.

Further studies with *Salmonella* strains expressing other heterologous antigens will be necessary to determine if prior *Salmonella* immunity generally suppresses or augments the antibody response to foreign antigens. It will also be necessary to examine if prior immunity affects the cellular response to foreign antigens as well as the antibody response. Longer-term studies in which the period between preimmunization and immunization is extended are needed to see if the detrimental effect on the immune response to the foreign antigen wanes with time.

Attenuated strains of *S. typhi* are being developed as improved oral typhoid vaccines and also as live carriers to deliver heterologous antigens. A number of stable attenuated *S. typhi* mutants have been constructed and are showing great promise in human volunteers. Several *S. typhi* strains that express FrgC have now been constructed and are awaiting human trials (7, 22). Our findings have serious implications for the development of a *Salmonella*-based oral tetanus vaccine. For a number of reasons described above, such a vaccine would be ideal for immunizing against tetanus in the developing world. Unfortunately, the people living in such areas may have been exposed to *S. typhi*. If our findings on the negative effect of prior *Salmonella* immunity on the response to a *Salmonella*-carried foreign antigen also apply to humans, then the *S. typhi*-FrgC vaccine may not be efficacious in individuals living in areas
where typhoid is endemic. However, our results also suggest possible ways to overcome this problem. Although immunity to a heterologous serotype also reduce the effectiveness of the S. typhimurium carrier, the effect was much less than that seen in mice which were immune to S. typhimurium. It is possible that a carrier unrelated to S. typhi, for example, one derived from a paratyphoid strain, may be effective in individuals in areas where typhoid is endemic. We found that the more immuno-
genetic the strain, the lower the effects of prior immunity. By searching for new promoter and expression systems for FrgC, it may be possible to construct strains that are able to induce protective immunity even in the face of preexisting immunity to the carrier itself.

Existing immunity to tetanus toxin, or derivatives thereof, should not affect the ability of a Salmonella carrier expressing FrgC to induce anti-FrgC response. In fact, Chabalagoty et al. (5) found that the anti-tetanus toxoid serum antibody response was greater in mice that had been preimmunized with tetanus toxoid than in mice with no prior immunity to tetanus toxoid.

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