Evaluation of Two Salmonella typhimurium Hybrids as Challenge Organisms in a System for the Assay of Typhoid Vaccines

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A mouse-virulent Salmonella typhimurium hybrid (H42), which expresses the Salmonella typhi Vi antigen in addition to S. typhi O antigens 9 and 12, and a mouse-virulent S. typhimurium hybrid (H1), which expresses only the 9 and 12 antigens of S. typhi, were compared in their behavior as challenge organisms in a system developed to assay the protective capabilities of typhoid vaccines. Swiss-Webster white mice, vaccinated intraperitoneally with live Escherichia coli hybrids expressing the S. typhi O antigens 9 and 12, were significantly protected against death from intraperitoneal challenge with each of the S. typhimurium hybrid strains. Vaccination with an E. coli hybrid expressing the S. typhi Vi antigen in addition to O antigens 9 and 12 was seen to confer no advantage in protection against either S. typhimurium hybrid challenge organism over that obtained by vaccination with an E. coli hybrid expressing only the O antigens of S. typhi. However, a notable difference in the behavior of the two S. typhimurium hybrids was seen in mice vaccinated with the parent of the E. coli hybrid vaccinating strains, E. coli F464, which expresses no surface antigens common to either of these S. typhimurium hybrid challenge organisms. A nonspecific (with respect to the vaccinating strain) protective effect, believed to be associated with Vi antigen expression by the challenge organism, was seen against the challenge with S. typhimurium hybrid H42 after F464 vaccination, whereas no protection was conferred by F464 vaccination against the challenge with Vi-nonexpressing S. typhimurium hybrid H1. Inasmuch as neither S. typhimurium hybrid discriminates between the expression or nonexpression of the Vi antigen in a vaccinating strain, it is concluded that the Vi-nonexpressing S. typhimurium hybrid H1, which more clearly indicates the vaccine-specific protective role of the S. typhi O antigens and does not exhibit the nonspecific protection response of hybrid H42, is the better choice as challenge organism for this vaccine assay system.

In previous studies (1–5) aimed at developing an animal protection system suitable for the assay of vaccines against typhoid fever in humans, we have examined the feasibility of using mouse-virulent Salmonella typhimurium hybrids expressing Salmonella typhi surface antigens as challenge organisms in vaccinated Swiss-Webster white mice. Our initial expectation was that the ideal hybrid for this purpose would be one expressing a complete set of these S. typhi-derived antigens, i.e., the O antigens 9 and 12, the flagellar antigen d, and the Vi antigen. Such a hybrid is S. typhimurium H42, and, indeed, this strain has been employed as a challenge organism in every one of our previous studies with this assay system (1–5). However, several factors have become apparent during the course of these studies which suggest that an S. typhimurium hybrid expressing only the O antigens of S. typhi might be more suitable for this purpose than hybrid H42.

In an earlier study (1), we conducted experiments in which S. typhimurium hybrids, expressing different combinations of S. typhi-derived surface antigens, were used as challenge organisms in mice immunized with nonliving vaccines prepared from strains of S. typhimurium, S. typhi, and S. typhimurium hybrids that expressed the S. typhi surface antigens; the results of these experiments indicated that the immune response generated against the O surface antigens of the challenge organism is an important factor in determining the ability of the vaccine to protect the animals against death.
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This importance of the O antigen antibodies in the protection of mice against Salmonella infection was suggested earlier by the studies of Ornellas et al. (13) and has subsequently been confirmed by the work of Severson et al. (16) as well as that of Lyman et al. (12). However, our studies with these S. typhimurium hybrids have also indicated that only those immunogens directed against the O antigens of the challenge organism are involved in the vaccine-specific protection conferred in this system: no apparent protective role has been observed for immunogens against either the Vi antigen or the flagellar antigens expressed by the challenge organism (1, 3–5). Furthermore, one of our earliest studies suggested that expression of the Vi antigen by an S. typhimurium hybrid may alter its virulence characteristics in a manner which adversely affects its ability to respond properly as a challenge organism, with the result that some vaccines protect nonspecifically against such hybrids (1). An especially disturbing example of this nonspecific protective effect was seen in our administered intraperitoneally. Survivors were counted from toxemia seldom occurred. Mice were challenged with the Escherichia coli strain F464, which expresses no surface antigens common to S. typhimurium hybrid H42, were significantly protected against death after challenge with that hybrid. In view of these findings, a reconsideration of the merit of using hybrid H42 as a challenge organism in this system seemed in order. In the present communication, we compare the behavior of S. typhimurium hybrid H42 as a challenge organism with that of S. typhimurium hybrid H1, which expresses only the O surface antigens of S. typhi, and we argue that hybrid H1 is the better choice for vaccine assay.

MATERIALS AND METHODS

Bacterial strains. The male parent of all of the hybrids employed in this study is S. typhi WR4000 (serotype O9, 12; Vi; d), originally designated TD-7, an Hfr strain whose derivation and description were reported previously (8). Kiefer et al. (10) described the generation of E. coli hybrid F1061 from the mating between S. typhi WR4000 and E. coli F464 (serotype O8, K : H 1) as the consequence of recombination at the rfb loci. E. coli hybrid F1061 expresses the S. typhi O antigens 9 and 12 and lacks the O8 specificity of its E. coli parent F464. E. coli hybrid WR3078 was derived from E. coli hybrid F1061 by transfer of the Vi antigen-determining (viab+) genes from S. typhi WR4000 (3); E. coli hybrid WR3078 expresses the S. typhi Vi antigen in addition to S. typhi O antigens 9 and 12. The S. typhimurium hybrid challenge organisms H1 and H42, originally referred to as WR5004 His+, 9, 12; i-1, 2 and WR5004 His+, Ara-2, 9, 12, Vi; d-1, 2, respectively, were derived from the mouse-virulent S. typhimurium strain WR5004 by mating with S. typhi WR4000, as described in a previous communication (2). S. typhimurium hybrid H42 expresses the S. typhi O antigens 9 and 12, the S. typhi Vi antigen, and the S. typhi flagellar antigen d. whereas S. typhimurium hybrid H1 expresses only the 9 and 12 O antigens of S. typhi: each of these hybrids has a 50% mouse lethal dose of less than 50 organisms. The well-known S. typhi Ty2 strain (O9, 12; Vi; d) has been employed in our previous protection studies (1–5), and the S. typhi Ty2W strain is a Vi-nonexpressing derivative of Ty2.

Protection experiments. Organisms were harvested in phosphate-buffered saline, washed three times, and appropriately diluted. Swiss-Webster white mice (40 per group) HPB strain, random bred, and weighing 16 to 18 g were vaccinated intraperitoneally with 10^7 live organisms of the immunizing strain suspended in 0.5 ml of solution. (In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.) The animals tolerated this vaccinating dose well with all of the immunizing strains employed, and deaths resulting from toxemia seldom occurred. Mice were challenged 5 weeks later with 2,500 organisms (in 0.5 ml) of S. typhimurium hybrid H42 or S. typhimurium hybrid H1 administered intraperitoneally. Survivors were counted 21 days after challenge.

Test for statistical significance. The results of the assays were analyzed as RXC tables by using the usual chi-square criterion (15).

RESULTS

S. typhimurium hybrid H42, expressing the S. typhi surface antigens 9, 12, d, and Vi, has been used previously as a challenge organism in testing the live vaccinating strains E. coli hybrid F1061, E. coli hybrid WR3078, and their parent, E. coli F464 (3); its response to those strains in the present study was similar to that which we observed in the earlier study. S. typhimurium hybrid H1, which expresses only the 9 and 12 O surface antigens of S. typhi, has not been employed previously as a challenge organism with any of the live vaccinating strains examined here. The comparison of the behavior of hybrid H1 with that of hybrid H42, as challenge organisms in protection experiments with five live vaccinating strains, is shown in Table 1.

The notable difference in the behavior of the two S. typhimurium hybrid challenge organisms was seen in those mice vaccinated with the live E. coli strain F464. The nonspecific (with respect to the vaccinating strain) protective effect against hybrid H42 resulted in the survival of 50% of the F464-vaccinated animals challenged with this hybrid, which is a significant level of protection (P < 0.005). In contrast, similarly F464-vaccinated mice were not protected when S. typhimurium hybrid H1 was employed as the challenge organism.

E. coli hybrids expressing S. typhi O antigen specificity have been suggested as possible candidates for use in human subjects to develop a
TABLE 1. Comparison of the behavior of S. typhimurium hybrids H1 and H42 as challenge organisms in intraperitoneally vaccinated mice*

<table>
<thead>
<tr>
<th>Vaccinated with:</th>
<th>S. typhimurium H42 (O9, 12: Vi:d: 1, 2)</th>
<th>S. typhimurium H1 (O9, 12: Vi: 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivors/no. inoculated</td>
<td>% Survival</td>
<td>Survivors/no. inoculated</td>
</tr>
<tr>
<td>S. typhi Ty2 (O9, 12: Vi:d: 1, 2)</td>
<td>37/40</td>
<td>92.5</td>
</tr>
<tr>
<td>S. typhi Ty2W (O9, 12: d)</td>
<td>36/40</td>
<td>90.0</td>
</tr>
<tr>
<td>E. coli F1061 (O9, 12)</td>
<td>29/40</td>
<td>72.5</td>
</tr>
<tr>
<td>E. coli WR3078 (O9, 12: Vi)</td>
<td>28/40</td>
<td>70.0</td>
</tr>
<tr>
<td>E. coli F464 (O8)</td>
<td>20/40</td>
<td>50.0</td>
</tr>
<tr>
<td>Control (not vaccinated)</td>
<td>2/40</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* The vaccinating dose was 10⁷ live organisms, and the animals were challenged intraperitoneally after 5 weeks with 2,500 organisms of either S. typhimurium hybrid H1 or S. typhimurium hybrid H42.

** Surface antigens expressed by the vaccinating strains and the challenge strains are shown in parentheses.

Mortality is significantly less (P < 0.005) than in the control group.

safe, orally administered, vaccine for the prevention of typhoid fever (10). Vaccination here with either of the F464-derived live E. coli hybrids F1061 (expressing the S. typhi O antigens 9 and 12) or WR3078 (expressing the S. typhi O antigens 9 and 12 and the Vi antigen) conferred significant protection against both S. typhimurium hybrid challenge organisms. With hybrid H1 as the challenge organism, the protection resulting from S. typhi O antigen expression by the E. coli hybrid vaccinating strains was readily apparent when viewed in comparison with the absence of protection against H1 in the animals vaccinated with the E. coli parent strain F464. However, with H42 as the challenge organism, the influence of the S. typhi O antigens in those E. coli hybrid vaccinating strains was largely obscured by the nonspecific protective effect against hybrid H42. Vaccination with the Vi antigen-expressing (Vi-positive) E. coli hybrid WR3078 was seen to confer no protection advantage over vaccination with the Vi-nonexpressing (Vi-negative) E. coli hybrid F1061 against a challenge with either of the S. typhimurium hybrids. The best protection against both challenge organisms was conferred by vaccination with the live S. typhi strains Ty2 and the Vi-negative Ty2W. The level of protection conferred against H1 by these strains was about the same as that conferred against H42, and there was no essential difference between Ty2 and Ty2W in the protection conferred against either hybrid challenge organism.

DISCUSSION

The results of the present study indicate and reinforce several points that have been suggest-
We pointed out earlier (4) that our use of S. typhimurium WR5004 and its S. typhi surface antigen-expressing hybrid derivatives administered intraperitoneally at low dose levels, it was observed that all of the Vi-positive hybrids were measurably slower (2 to 3 days) in bringing about the onset of deaths in nonimmunized mice than were S. typhimurium WR5004 and Vi-negative hybrids of this strain (1; B. B. Diena, unpublished data). We also noted in the present study, this 2- to 3-day delay in the onset of mouse deaths in the nonvaccinated control group challenged with S. typhimurium hybrid H42. Earlier (1), we speculated that this altered virulence pattern might be a factor in the protective effect of nonspecific vaccination against these Vi-positive hybrid challenge strains. At the present time, however, we are still not able to provide a fully satisfactory explanation for this effect. Nevertheless, we consider the protection conferred against S. typhimurium hybrid H42 by vaccination with E. coli F464, which expresses no surface antigens common to that hybrid, to be an improper response for a system intended to differentiate vaccines on the basis of their antigenic specificity and to reflect their potential as immunizing agents against typhoid fever in humans. The use of S. typhimurium hybrid H1 in the present comparison provides what we consider a more appropriate response, i.e., no significant protection is conferred by E. coli F464, and its hybrid derivatives are protective because of their expression of the S. typhi O antigens. In previous studies with this system (1, 3-5), we have repeatedly observed the inability of vaccination with the Vi antigen to influence protection against S. typhimurium hybrid H42 or any other Vi-expressing S. typhimurium hybrid challenge organisms that we have examined. This has been the case regardless of whether the Vi antigen was administered to the animals intraperitoneally (1, 3-5) or orally (3, 5), in purified form (4, 5) or as a component of nonliving vaccinating organisms (1), or, as in the present study, as a component of live vaccinating strains (3). Inasmuch as S. typhimurium hybrid H42 is also seen to be less discriminating as a challenge organism than S. typhimurium hybrid H1, there seems to us no point in further employment of H42 for this purpose. Therefore, some consideration seems in order as to whether using Vi-negative S. typhimurium hybrid H1 as a challenge organism in the present mouse assay system can, in fact, provide a valid indication of the potential of a vaccine for preventing typhoid fever in humans.

We pointed out earlier (4) that our use of S. typhimurium hybrids expressing S. typhi antigen, as challenge organisms in the present system, has provided a different answer to the question of which S. typhi antigens are important in mouse protection than has the use of mucin-treated S. typhi as the challenge organism. In the latter system, which is currently employed as a laboratory assay for typhoid vaccines, the protective role of the Vi antigen has been well documented (11, 14, 19). However, neither the importance of the Vi antigen in mouse protection in that system nor its lack of involvement in protection in ours provides a convincing indication of the role of this antigen in human protection. As we have frequently stated, evidence for the role of any antigen in protection against typhoid fever in humans must come from studies conducted with human subjects. Accordingly, the desired assay system is one in which the laboratory test results are in agreement with the findings of human experience.

Although it has long been assumed by many workers that the Vi antigen is important in the protective immunization of humans against typhoid fever, there has never been, to our knowledge, any experimental evidence, either from field trials or volunteer studies, to support this belief. On the other hand, studies in recent years with the attenuated S. typhi galE mutant Ty21a (6, 7, 17, 18) have shown that effective human typhoid protection can be achieved by an immunization in which the Vi antigen is not involved at all. S. typhi Ty21a is Vi negative (7; our own observation). Nevertheless, when administered as a live, oral vaccine, Ty21a has been shown to be highly effective in protecting against typhoid fever, both in human volunteers (7) and in a recently concluded field trial that involved over 32,000 school children in Alexandria, Egypt (17, 18). Importantly, statistically significant protection is conferred by S. typhi Ty21a when the organism is grown under conditions in which its O antigens are synthesized, but not when it is grown under conditions in which its O antigens are not synthesized (7). Thus, in addition to showing that the Vi antigen is not required for protection, the behavior of this strain also indicates the importance of the S. typhi O antigens in human typhoid immunization.

At the present time, the only example we know of in which the Vi antigen can clearly be demonstrated to play a role in protective immunization is the standard mouse assay system for typhoid vaccines, in which S. typhi, an obligate human pathogen, is manipulated to serve, artificially, as a challenge organism. Although artificiality will always exist to some extent in any model system, our assay does utilize, we believe, a somewhat more natural infection model, in that the hybrid challenge strain is derived...
from an organism that is actually a mouse pathogen. We think, therefore, that the absence of a detectable protective role for the Vi antigen in this system merits some consideration. This consideration is further warranted, we believe, by the continuing absence of any experimental evidence that might demonstrate a protective role for the Vi antigen in human typhoid immunity. If, as we suspect, the most effective vaccines for human typhoid immunization are seen to be live, avirulent, orally administered organisms such as S. typhi Ty21a, whose protective capability is dependent upon expression of the S. typhi O antigens, and not upon the Vi antigen, we believe that the use of S. typhimurium hybrid H1 as a challenge organism in the presently studied system will produce test results that are in line with those of human experience.

LITERATURE CITED
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