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Expression of immunogenic epitopes of hepatitis B surface antigen with hybrid flagellin proteins by a vaccine strain of *Salmonella*

(hepatitis B virushepatitis B vaccine)

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ABSTRACT **A nonvirulent *Salmonella dublin* flagellin-negative, aromatic-dependent live vaccine strain has been used to express hepatitis B virus surface antigen epitopes in an immunogenic form. The envelope proteins of the virion are encoded by the S gene, which contains the pre-S_1, pre-S_2, and S coding regions. Synthetic oligonucleotides corresponding to amino acid residues S-(122–137) and pre-S_2-(120–145) were inserted in-frame into the hypervariable region of a cloned *Salmonella* flagellin gene, and the recombinant plasmids were introduced into a flagellin-negative *aroA* mutant live vaccine strain of *S. dublin*, SL5928. The flagellin gene was expressed in bacteria carrying the plasmids as detected by immunoblotting with anti-flagellin (H1-d) serum. Both the S and pre-S_2 epitopes were detected in bacteria carrying the relevant plasmid by immunoblotting with anti-HBs (antibody to hepatitis B virus surface antigen) and anti-peptide antisera. Animals immunized intramuscularly or orally with the live recombinant bacteria developed antibodies specific to these hepatitis B virus epitopes as detected by ELISA.**

We have investigated a general vaccine strategy in which viral antigens are expressed in live attenuated vaccine strains of *Salmonella* to determine whether immune responses to viral antigens as well as *Salmonella* antigens can be achieved when such live bacteria are used for oral vaccination. Hepatitis B virus (HBV) has been used to investigate this approach because of the extensive knowledge of its antigenic structure and of epitopes that appear to be able to elicit protective immunity.

The envelope proteins of the hepatitis B virion contain the hepatitis B surface antigen (HBsAg) (1). The most prevalent viral structures in the serum of chronic HBsAg carriers are small incomplete viral particles that contain HBsAg polypeptides embedded in spherical or filamentous membrane structures (2). The S gene contains three coding regions (pre-S_1, pre-S_2, and S) specifying respective peptide sequences; each contain multiple epitopes present on the surfaces of these particles. HBsAg particles derived from human plasma or HBsAg particles produced by recombinant DNA methods (some of which lack pre-S epitopes) have been shown to elicit a protective immune response, and the purified particles represent current vaccines for HBV (3).

Peptides consisting of amino acid sequences predicted from nucleotide sequences of pre-S and S regions have been synthesized in order to localize HBsAg determinants (4–7). Both group-reactive *a* specificity and subtype *d/y* specificity have been found in synthetic peptides corresponding to amino acid residues S-(110–139) (7, 8). Chimpanzees immunized with synthetic peptide S-(110–137) produced subtype-specific IgM anti-HBs and were partially or completely protected from the live virus challenge despite the transient nature of the antibody responses (7). pre-S_2 peptides have also been protective in chimpanzees. Antibody produced in rabbits against synthetic peptide pre-S_2-(120–145) neutralized HBV infectivity (9), and immunization with synthetic peptide pre-S_2-(133–151) protected chimpanzees from subsequent challenge with infectious HBV (10). Studies in mice have shown that peptide pre-S_2-(120–145) contains nonoverlapping recognition sites for both B and T lymphocytes (11–13). The B-cell epitope was near the carboxyl terminus [pre-S_2-(133–145)] and reacted with the native HBsAg/p33, whereas the T-cell epitope was near the amino terminus [pre-S_2-(120–133)] and provided helper function in S-nonresponder mice (13). Immunization with synthetic peptides corresponding to several regions of pre-S_1 or pre-S_2 have been shown to induce T-helper activity for anti-S responses in mice. Whether the epitopes identified in mice serve similar functions in humans has not been determined.

The *Salmonella* gene *H1* is the structural gene for flagellin, the major polypeptide of the filament part of *Salmonella* flagella with phase 1 antigenic character. The base sequence of four *H1* alleles determining different flagellar antigens has revealed a hypervariable 350-base-pair (bp) central region (14). When a 48-bp *Eco*RV–*Eco*RV fragment in the hypervariable region of gene *H1-d* was deleted and a synthetic oligonucleotide encoding a cholera toxin epitope was inserted, the epitope was detected and was immunogenic in chimeric flagellin expressed in an attenuated strain of *Salmonella* (S.N., C. O. Jacob, and B.S., unpublished results). Such *Salmonella* sp. strains made aromatic-dependent by non-reverting *aroA* mutation are nonvirulent (15) and have been found to be safe and effective as a live vaccine in mice, calves, and sheep (15–17).

In this study, we have inserted two specific HBV S gene sequences encoding, respectively, peptides S-(122–137) and pre-S_2-(120–145) into the *Salmonella* flagellin gene *H1-d*, and HBsAg epitopes were shown to be expressed by an attenuated *Salmonella dublin* strain carrying the recombinant plasmids. Immunization of animals with live bacteria led to both anti-HBs and anti-flagellin responses.

MATERIALS AND METHODS

Synthetic Oligonucleotides, Synthetic Peptides, and Recombinant DNA Methods. Synthetic oligonucleotides with specific sequences (see Fig. 1) were purified by PAGE. The corresponding peptides S-(122–137) and pre-S_2-(120–145) were synthesized by the solid-phase method of Erickson and Merrifield (18) and purified on Sephadex LH-20; the purity was checked by analytical reverse-phase HPLC and amino acid analysis. Bacterial lysates were prepared from 1.0 ml of overnight cultures by centrifuging bacteria and resuspending

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

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them in 0.1 ml of sample buffer containing 2% (wt/vol) SDS (Sigma) and 2% (vol/vol) 2-mercaptoethanol (Sigma), as well as phenylmethanesulfonyl fluoride, N^{α}-(*p*-tosyl)lysine chloromethyl ketone, L-1-tosylamido-2-phenylethyl chloromethyl ketone, leupeptin, and pepstatin (the protease inhibitors were purchased from Boehringer Mannheim) at appropriate concentrations.

Antisera. A polyclonal rabbit anti-H1-d (*Salmonella* phase 1 flagellin antigen) serum was from P. H. Makela (Helsinki). Polyclonal goat anti-HBs (raised against HBsAg purified from human plasma) was from Dako (Santa Barbara, CA). Antisera against peptides S-(122–137) and pre-S_2-(120–145) conjugated with thyroglobulin were raised in guinea pigs. Optimal dilutions were used to detect respective HBsAg epitopes in bacterial lysates by immunoblotting.

Immunization. Bacterial clones for immunization were grown overnight at 37°C in Luria broth containing ampicillin at 50 mg/ml. Cells were washed twice and resuspended in phosphate-buffered saline. Two New Zealand White rabbits were immunized intramuscularly with $\approx 10^9$ live bacteria of each clone in 1 ml on days 0, 7, 14, 21, and 28; blood was taken on days 0, 28, 56, and 84.

Three guinea pigs and 10 mice [known responder strains, B10.BR for pre-S_2-(120–145) clones and BALB/cj for S-(122–137) clones] (13) were orally immunized with $\approx 10^9$ live bacteria of each clone in 1 ml for each guinea pig and approximately 5×10^8 live bacteria in 0.05 ml for each mouse on days 0, 7, 14, and 28; blood was taken on days 0, 28, 56, and 84.

Sera were assayed for specific antibodies by ELISA (19) with alkaline phosphatase-conjugated anti-rabbit, anti-mouse, or anti-guinea pig antisera from Boehringer Mannheim. The titer refers to the highest dilution of test serum at which the ratio of A_{405} of test serum to the A_{405} of preimmune serum was >2.0.

RESULTS

Construction of Recombinant Plasmids. Synthetic oligonucleotides each encoding an HBsAg (subtype ayw) amino acid sequence that appears to contain a protective or partially protective epitope were used [S-(122–137) and pre-S_2-(120–145)]. The upper lines in Fig. 1 represent the nucleotide sequences of the corresponding synthetic oligonucleotides that were designed for insertion in-frame into the *Eco*RV sites of the flagellin gene (Fig. 1). The codons chosen were the most frequently used ones in the *Salmonella* flagellin gene (14). Restriction sites for *Kpn* I and *Bam*HI [for the S-(122–137) and pre-S_2-(120–145) coding sequences, respectively] were included to allow identification of recombinants by restriction analysis. A half site for *Eco*RV was put at the 3′ end of the pre-S_2 oligonucleotide to facilitate ligation with oligonucleotides for other HBV epitopes (see *Discussion*). Stop codons in different reading frames (underlined) were placed in the noncoding strands for easy selection of clones with inserts in the desired orientation. The flagellin gene was contained in plasmid pLS405 consisting of a 3.8-kilobase *Salmonella muenchen* genome fragment containing the 1.5-kilobase flagellin coding sequence cloned into the *Eco*RI site of plasmid pUC19. The central hypervariable region of the wild-type flagellin gene contains two in-frame *Eco*RV sites 48 bp apart (Fig. 1). Deletion of this *Eco*RV fragment in pLS405 reduces but does not abolish the flagellation of bacteria (S.N., unpublished work). Overlapping complementary single-stranded synthetic oligonucleotides were hybridized, phosphorylated, repaired with the Klenow fragment of the *Escherichia coli* DNA polymerase to make blunt-end double-stranded DNA fragments, and ligated into *Eco*RV site of pLS405 with T4 DNA ligase; the ligation reaction mixture was used to transform an *E. coli* C600 *hag*$^-$ variant, which was flagellin-negative (CL447). Clones with recombinant

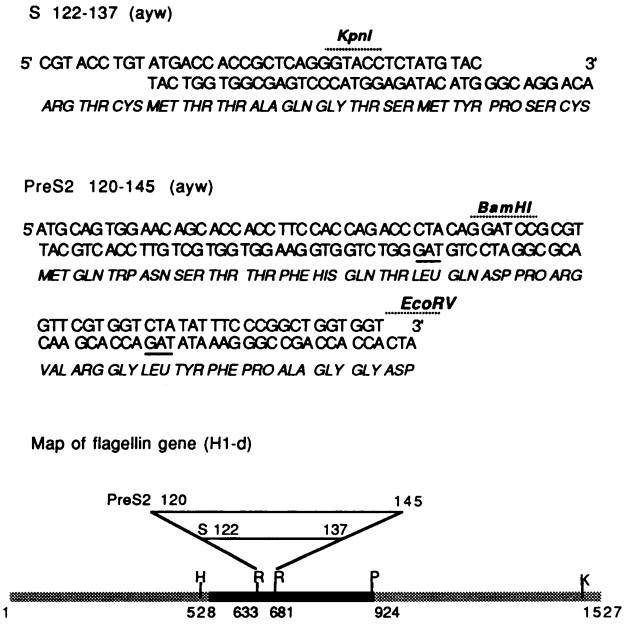

FIG. 1. Amino acid and synthetic oligonucleotide sequences of HBsAg (ayw) S-(122–137) and pre-S_2-(120–145) and map of the flagellin gene. The solid bar represents the hypervariable region. H, *Hin*dIII; R, *Eco*RV; P, *Pst* I; K, *Kpn* I. Numbers below the map are in base pairs.

plasmids were identified by colony hybridization using the respective synthetic oligonucleotide labeled with ^{32}P as probe and by restriction digestion. The number, orientation, reading frame, and fidelity of inserts were determined by dideoxynucleotide sequencing (20) by using a 15-nucleotide synthetic primer corresponding to a flagellin gene sequence about 30 bp downstream of the *Eco*RV site. Several recombinant plasmids with 1–3 copies of the respective synthetic oligonucleotide sequence in different orientations were isolated and further characterized (Fig. 2).

Characterization of Recombinant Clones. The recombinant plasmids were used to transform *Salmonella typhimurium* LB5000 (a restriction-negative, modification-proficient, nonflagellated strain with mutation *flaA66*) competent cells and were then transferred to a flagellin-negative live vaccine strain of *S. dublin* SL5928 by transduction using phage (P22 HT105/1 int), with selection for ampicillin resistance in each case. SL5928 is an aromatic-dependent strain derived from *S. dublin* SL1438 (21), and it is nonmotile because it is monophasic with its single flagellin gene inactivated by a transposon, Tn*10*, insertion. Antigens expressed in both the *E. coli* C600 *hag*$^-$ variant strain and SL5928 were examined by immunoblotting with either rabbit anti-H1-d, goat anti-HBsAg, or anti-synthetic pre-S_2-(120–145) peptide antisera (Fig. 3). Bacterial lysates were heated to 100°C for 3 min before loading; proteins were separated by SDS/12.5% PAGE (22), then transferred to nitrocellulose filters, and immunostained with the desired antiserum. As expected, no flagellin antigen was detected in the *E. coli* C600 *hag*$^-$ variant or *S. dublin* SL5928, which do not have an intact flagellin gene (Fig. 3*A*, lanes 1 and 3). Two electrophoretic components reacting as a flagellin antigen were detected in clone S20 (Fig. 3*A*, lanes 6 and 8), and the basis of this complex pattern is not clear. The goat anti-HBs antisera reacted with the hybrid flagellin proteins of clones S16 and S20 [with the sequence encoding S-(122–137)] and clones pS8 and pS21 [with the sequence encoding pre-S_2-(121–145) and pre-S_2-(120–145), respectively], indicating that hybrid proteins of these clones contained epitopes recognized by this anti-HBs-containing serum. Rabbit anti-pre-S_2-(120–145) peptide reacted with the hybrid flagellin

Constructs	no.	Bacterial motility	Ab binding by Western blot: anti-F	anti-HBs	anti-S 122-137	anti-preS2 120-145	Ab Response detected by ELISA with syn. pep.: S122-137	preS2 120-145
S122-137	S16	-	+	+	+	-	+	-
	S20	+	+	+	+	-	+	-
	S6	+	+	-	-	-	nd	nd
	S27	+	+	-	-	-	nd	nd
preS2 121-145	pS8	-	+	+	-	+	-	+
preS2 120-145	pS21	-	+	+	-	+	-	+
	pS2	-	-	-	-	-	nd	nd
	pUC-F	+	+	-	-	-	-	-

FIG. 2. Characteristics of cloned plasmid pLS405 recombinants. The solid (for S sequences) or hatched (for pre-S_2 sequences) arrows represent the orientation and number of synthetic oligonucleotide sequences inserted between the *Eco*RV sites of the flagellin gene with respect to the orientation of the flagellin gene (represented by the hatched arrows) of pLS405. Bacterial motility was tested using semisolid agar. Rabbits were immunized with *S. dublin* SL5928 transformed with individual plasmids, and the antibody responses were measured by ELISA using each synthetic peptide as coating antigen. nd, Not done; anti-F, anti-flagellin; syn. pep., synthetic peptide.

proteins of clones pS8 and pS21, which contain the pre-S_2 sequence (Fig. 3*B*, lanes 4–7), but not with the proteins of any of the clones with the S sequence (Fig. 3*B*, lanes 8–11).

The assembly of recombinant flagellin carrying HBsAg epitopes was investigated by electron microscopy of bacteria expressing the hybrid flagellin. In the motile clone, S20, flagella with morphology indistinguishable from that of wild-type flagella were seen on most bacteria. In the three nonmotile (as judged by ability to spread on semisolid agar) but flagellin-positive clones S16, pS8, and pS21, few flagella were observed (data not shown).

Fig. 2 summarizes characteristics of several recombinant clones that have been analyzed. As expected, only clones with S or pre-S_2 coding sequences in the same orientation as the flagellin gene (S16, S20, pS8, and pS21) led to expression of hybrid flagellin proteins with detectable S or pre-S_2 determinants. Among the small number of clones examined, only three of the four with an insert in an orientation opposite to that of the flagellin gene (S20, S6, and S27) were motile. Those with one or more inserts in the same orientation and no insert in the opposite orientation (S16, pS8, and pS21) did not spread in semisolid medium, although scanty flagella

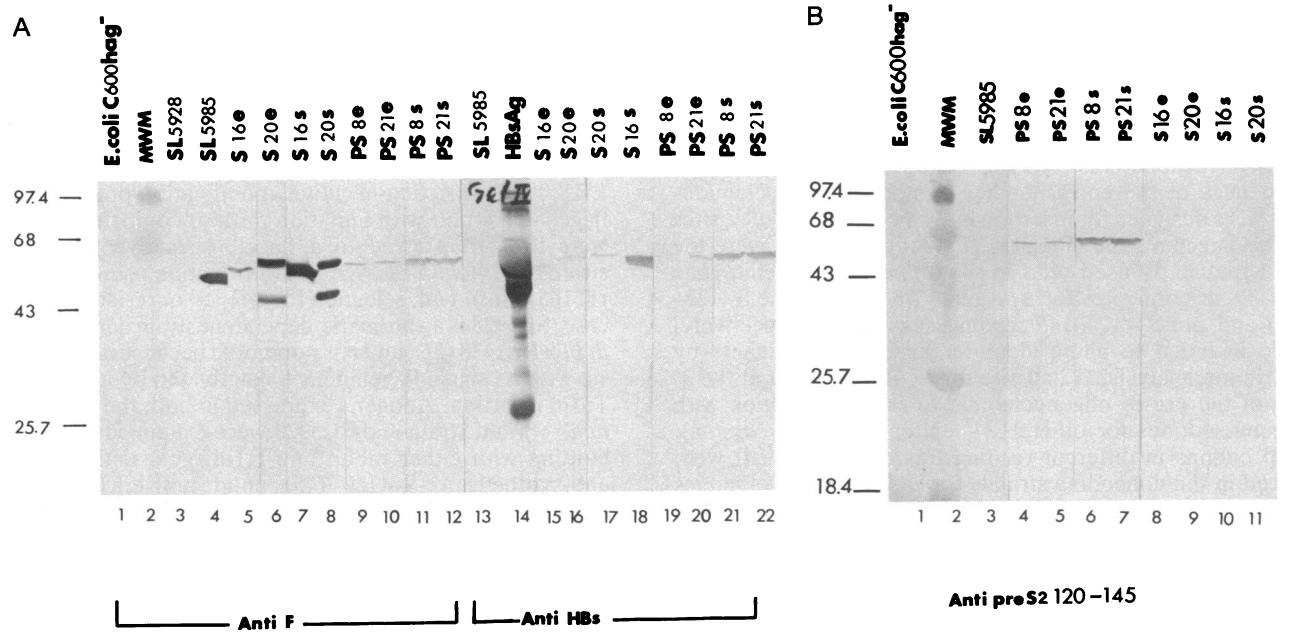

FIG. 3. Immunoblot analysis of the bacterial clones transformed with recombinant and control plasmids. The following were analyzed by SDS/12.5% PAGE: lysates of the *E. coli* C600 *hag*$^-$ variant transformed with recombinant plasmids containing sequences encoding S-(122–137) (S16e and S20e), the sequence for pre-S_2-(121–145) (pS8e), or the sequence for pre-S_2-(120–145) (pS21e); lysates of *S. dublin* SL5928 containing the same plasmids described above (S16s, S20s and pS8s, and pS21s, respectively); controls consisting of lysates of the untransformed *E. coli* C600 *hag*$^-$ strain and *S. dublin* SL5928 (lanes 1 and 3 in *A* and lane 1 in *B*) and *S. dublin* SL5985, which is SL5928 transformed with only the parent plasmid pLS405 with deletion of the *Eco*RV fragment (lanes 4 and 13 in *A* and lane 3 in *B*); molecular weight markers (MWM, lane 2); and HBsAg from a patient serum (lane 14 in *A*). The proteins were transferred to nitrocellulose filters and were immunostained with either rabbit anti-flagellin H1-d antiserum indicated as anti-F (lanes 1–12 in *A*) or goat anti-HBsAg (lanes 13–22 in lane *A*) or rabbit anti-synthetic peptide pre-S_2-(120–145) (*B*). Incubation with an appropriate second antibody conjugated with either alkaline-phosphatase (*A*, lanes 1–12 and *B*) or peroxidase (*A*, lanes 13–22) was followed by reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (for alkaline phosphatase) or diaminobenzidine/H_2O_2 (for peroxidase).

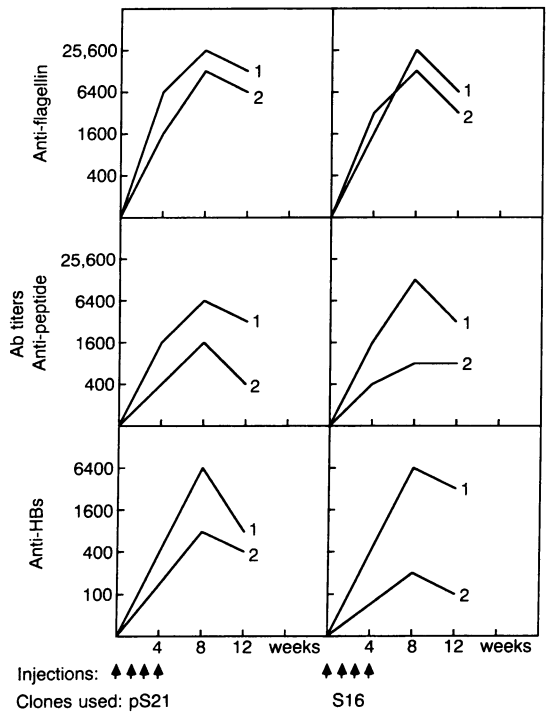

FIG. 4. Antibody responses of rabbits immunized intramuscularly with live *S. dublin* SL5928 transformed with S16 or pS21. Anti-flagellin indicates antibody detected by ELISA with the native flagellin protein purified from SL5928 transformed with the plasmid containing the wild-type flagellin gene, anti-peptide represents antibodies detected by ELISA with synthetic peptides S-(122–137) and pre-S_2-(120–145) (for animals immunized with SL5928 carrying S16 and pS21, respectively), and anti-HBs represents antibody detected by ELISA with HBsAg produced in Chinese hamster ovary cells. Ab, antibody.

were detected by electron microscopy and both flagellin and HBsAg epitopes were detected by immunoblotting.

Immunogenicity of Bacteria Expressing Hybrid Proteins. The immunogenicity of HBsAg epitopes in the hybrid flagellin proteins was first tested by intramuscular immunization of rabbits with live *S. dublin* SL5928 expressing hybrid flagellin (Fig. 4). High titers (above 10^4) of anti-flagellin antibodies were elicited in the two animals receiving SL5928 transformed with plasmid S16 [with the sequence encoding S-(122–137)] and in the two receiving SL5928 transformed with plasmid pS21 [with the sequence encoding pre-S_2-(120–145)]. The ELISA titers of anti-peptide antibodies [anti-S-(122–137) or anti-pre-S_2-(120–145) in the respective rabbits immunized with SL5928 carrying the corresponding plasmid] varied between 10^3 and 2×10^4. These antisera reacted with recombinant HBsAg (subtype ayw and containing pre-S_2 sequence) produced in Chinese hamster ovary cells (kindly provided by P. Tiollais of Institut Pasteur) (23) with peak titers of ≈6400 in two of four rabbits. The immune sera from these rabbits also reacted strongly with native HBsAg purified from HBV-infected chimpanzees detected by Abbott Laboratory's Ausab assay (data not shown). Rabbits immunized with SL5928 transformed with plasmids S20 and pS8, respectively, responded similarly (data not shown) to the animals immunized with SL5928 containing S16 and pS21. In two rabbits immunized with SL5928 containing the parental plasmid pLS405 without insertion of HBV sequences, high levels of anti-flagellin antibody were detected as expected and no anti-S or anti-pre-S_2 peptide or anti-HBsAg antibodies were detected. None of the animals inoculated with this attenuated *S. dublin* mutant (SL5928) manifested signs of septic shock or other illness. These results indicate that the hybrid flagella expressed by *S. dublin* SL5928 carrying the recombinant plasmid contain HBsAg epitopes that are immunogenic and that antibody elicited by them reacts with plasma-derived or recombinant HBsAg.

Synthetic peptides S-(122–137) and pre-S_2-(120–145) specifically blocked the binding of HBsAg produced in Chinese hamster ovary cells by antibodies in the immune sera but not the preimmune sera (data not shown) of rabbits immunized with SL5928 clones expressing the S or pre-S_2 epitopes, respectively, confirming that the anti-HBs in these animals was directed at epitopes encoded by the sequences introduced into the flagellin gene.

To determine whether anti-HBs responses would result from oral administration of live attenuated *S. dublin* SL5928 expressing hybrid flagella, experiments were carried out in rabbits, mice, and guinea pigs. Fig. 5 shows anti-peptide and anti-HBs titers in mice after oral vaccination with SL5928 transformed with each of the recombinant plasmids S16, S20, pS8, and pS21 and with the unaltered flagellin plasmid pLS405. Significant titers of the respective anti-peptide and anti-HBs were detected in all animals, although the titers were lower than those observed after intramuscular immunization of rabbits. Oral administration of pLS405-transformed bacteria SL5928 resulted in no detectable anti-HBs or anti-peptide antibody as expected. The titers of anti-peptide and anti-HBs in rabbits and guinea pigs (data not shown) were similar (80–640) to those in mice (Fig. 5) after oral administration of live *S. dublin* SL5928. No diarrhea or other disease manifestations were observed in any animals given *S. dublin* SL5928 orally. These experiments indicate that immune responses to HBsAg epitopes are elicited by oral vaccination with live *S. dublin* SL5928 expressing hybrid flagella.

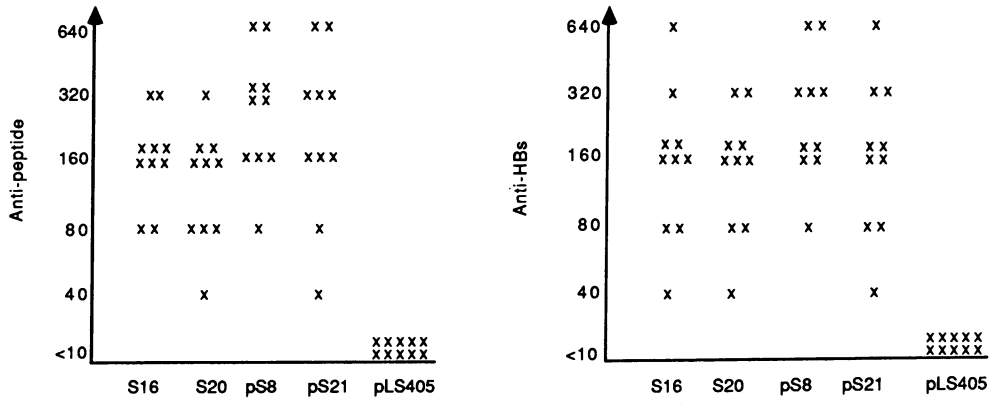

FIG. 5. Antibody responses in the mice immunized orally with live SL5928 expressing an HBsAg epitope. Each "x" represents the antibody titer of an individual mouse.

DISCUSSION

We have shown that nucleotide sequences encoding antigenic regions of HBsAg polypeptides can be inserted into the hypervariable region of the *Salmonella* flagellin gene and that these sequences can be expressed in an attenuated Salmonella mutant. Some resulting hybrid flagellin proteins can be assembled into functional and others into nonfunctional flagella as tested by ability to spread on semisolid medium. The hybrid flagella contain both flagellin and HBsAg epitopes detected by immunoblotting. The HBsAg epitopes were detected with antisera raised against specific synthetic peptides and against serum-derived HBsAg. Clearly, the number and orientation of HBsAg sequences inserted into the flagellin gene affected the function of assembled flagella. Interestingly, a HBsAg sequence inserted in the same (and not in the opposite) orientation as the flagellin gene reduced bacterial motility, suggesting that the specific viral envelope protein sequence [S-(122–137)] replacing a natural flagellin sequence of the same size significantly altered the conformation of the hybrid flagellin. In addition, replacing the 16-amino acid flagellin deletion with a 27-amino acid insert [pre-S_2-(120–145)] did not prevent expression of flagellin. In both, HBsAg epitopes recognized by antisera to native HBsAg were detected in the hybrid flagellin protein. These sequences as presented by live bacteria were immunogenic and elicited antibody that recognized native HBsAg. Thus flagellin represents a bacterial protein in which viral antigens can be presented in a form that is immunogenic in live vaccine strains of *Salmonella*.

It will be important to study the cellular immune responses to antigens presented in this way since cellular immunity may be important in protection against hepatitis B. Studies have shown protection of chimpanzees against HBV challenge after immunization with hepatitis B core antigen (or e antigen) (24, 25) may have involved cellular immune responses against these viral antigens. It will also be important to further investigate HBV pre-S and core polypeptides to identify epitopes that are most strongly recognized by T cells and B cells of humans as has been done in mice (12, 13). Studies of mucosal immune responses to viral antigens delivered by this method are important because mucosal immunity may offer optimum protection against pathogens that produce diseases on mucosal surfaces, and this vaccine strategy may effectively stimulate mucosal immunity and thus may be particularly suited to such agents.

In our experiments, serum anti-HBs responses were greater after intramuscular than after oral immunization when approximately the same number of bacteria were administered by each route. This difference could be due to the large fraction of orally administered bacteria that is excreted in the feces and the low number of organisms that enter tissue sites and become available to the immune system. Oral immunization may also suppress the systemic immune responses (26, 27). However, effective cellular responses may develop in the animals immunized orally despite low level of antibody response, as shown by protection against malaria in mice by oral immunization with a live vaccine strain of *Salmonella* expressing the circumsporozoite protein without detectable antibody (28).

The invasive properties of the bacterial strain are also important. Bacteria that only colonize the alimentary tract would not be expected to be strongly immunogenic after oral administration. Some degree of tissue invasion is considered essential for live oral vaccine strains of *Salmonella* to be effective immunogens (29). The degree of attenuation of any mutant strain of a pathogenic enteric organism will result not only in changes in virulence but also in immunogenicity by means of the oral route. Thus a critical factor in success or failure of this vaccine strategy in humans will be the availability of appropriately attenuated strains of *Salmonella* (or other pathogenic enteric bacteria) that are still sufficiently immunogenic in humans to provide a protective immune response to expressed HBV (or other viral) antigens.

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1. Tiollais, P., Pourcel, C. & DeJean, A. (1985) *Nature (London)* **317,** 489–495.
2. Heerman, K. H., Goldman, U., Schwartz, W., Seyffath, T., Baumgarten, H. & Gerlich, W. H. (1984) *J. Virol.* **52,** 396–402.
3. Krugman, S. (1982) *J. Am. Med. Assoc.* **247,** 2012–2015.
4. Lerner, R. A., Green, N., Alexander, H., Liu, F. T., Sutcliffe, G. & Shinnick, T. M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3403–3407.
5. Hopp, T. P. & Wood, K. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3824–3828.
6. Dressman, G. R., Sanchez, Y., Ionescu-Matiu, I., Sparrow, J. T., Six, H. R., Peterson, D. L., Hollinger, F. B. & Melnick, J. L. (1982) *Nature (London)* **295,** 158–160.
7. Gerin, J. L., Alexander, H., Shih, J. W.-K., Purcell, R. H., Dapolito, G., Engle, R., Green, N., Sutcliffe, J. G., Shinnick, T. M. & Lerner, R. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2365–2369.
8. Kennedy, R. C., Dressman, G. R., Sparrow, J. T., Culwell, A. R., Sanchez, Y., Ionescu-Matiu, I., Hollinger, F. B. & Melnick, J. L. (1983) *J. Virol.* **46,** 653–655.
9. Neurath, A. R., Kent, S. B. H., Parker, K., Price, A. M., Strick, N., Brotman, B. & Sproul, P. (1986) *Vaccine* **4,** 35–39.
10. Itoh, Y., Taka, E., Ohnuma, H., Kitjima, K., Tsuda, F., Machida, A., Mishiro, S., Nakamura, T., Miyakawa, Y. & Mayumi, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9174–9178.
11. Milich, D. R., Thornton, G. B., Neurath, A. R., Kent, S. B., Michel, M. L., Tiollais, P. & Chisari, F. W. (1985) *Science* **228,** 1195–1199.
12. Milich, D. R., McLanchlan, A., Chisari, F. V., Kent, S. B. & Thornton, G. B. (1986) *J. Immunol.* **137,** 315–322.
13. Milich, D. R., McLachlan, A., Chaisari, F. V. & Thornton, G. B. (1986) *J. Exp. Med.* **164,** 532–547.
14. Wei, L. N. & Joys, T. M. (1985) *J. Mol. Biol.* **186,** 791–803.
15. Hoiseth, S. K. & Stocker, B. A. D. (1981) *Nature (London)* **291,** 238–239.
16. Robertsson, J. A., Lindberg, A. A., Hoiseth, S. K. & Stocker, B. A. D. (1983) *Immunology* **41,** 742–746.
17. Nukkur, T. K. S., McDowell, J. H., Stocker, B. A. D. & Lascelles, A. K. (1986) *J. Med. Microbiol.* **24,** 11–15.
18. Erickson, B. W. & Merrifield, R. B. (1976) in *The Proteins*, eds. Neurath, H. & Hill, R. L. (Academic, New York), Vol. 2, pp. 255–258.
19. Gooderham, K. (1984) in *Methods in Molecular Biology*, ed. Walker, J. M. (Hummang Press, Clifton, NJ), Vol. 1, pp. 165–167.
20. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
21. Smith, B. P., Reina-Guerra, M., Stocker, B. A. D., Hoiseth, S. K. & Johnson, E. (1984) *Am. J. Vet. Sci.* **45,** 2231–2234.
22. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
23. Michel, M. K., Sobczak, E., Malpiece, R., Tiollais, P. & Streeck, R. E. (1985) *BioTechnology* **3,** 561–564.
24. Tabor, E. & Gerety, R. J. (1984) *Lancet* **i,** 172–174.
25. Murray, K., Bruce, S. A. & Hinnen, A. (1984) *EMBO J.* **3,** 645–649.
26. Mattingly, J. A., Kaplan, J. M. & Janeway, C. A., Jr. (1980) *J. Exp. Med.* **152,** 545–555.
27. Richman, L. K., Graeff, A. S., Yarchoan, R. & Strober, W. (1981) *J. Immunol.* **126,** 2079–2083.
28. Sardoff, J. C., Ballou, W. R., Baron, L. S., Majarian, W. R., Berry, R. N., Hockmeyer, W. T., Young, J. F., Cryz, S. J., Ou, J., Lowell, G. H. & Chulay, J. D. (1988) *Science* **240,** 336–338.
29. Germanier, R. & Furer, E. (1975) *J. Infect. Dis.* **131,** 553–557.