INFECTION AND IMMUNITY, Sept. 1974, p. 541-545

Vol. 10, No. 3
Printed in U.S.A.

Effect of Synthetic Disaccharide-Protein Conjugate as an Immunogen in *Salmonella* Infection in Mice

A. A. LINDBERG, L. T. ROSENBERG, Å. LJUNGGREN, P. J. GAREGG, S. SVENSSON, AND N.-H. WALLIN

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305; and Department of Bacteriology, National Bacteriological Laboratory, and Institute of Organic Chemistry, University of Stockholm, S-10405 Stockholm, Sweden

Received for publication 29 April 1974

The synthetic disaccharide 3-*O*-α-tyvelopyranosyl-α-D-mannopyranoside, identical to the O antigen 9 determinant in *Salmonella*, covalently linked to bovine serum albumin and suspended in Freund complete adjuvant, was used as an immunogen in mice. Antibodies in high titer were formed against the O9 determinant as estimated by a bactericidal assay, whereas only low titers of hemagglutinating antibodies were found. A sensitive in vivo assay demonstrated that in immunized mice, after challenge with two strains of equal virulence but with different O antigens (O9,12 and O4,5,12, respectively), the growth of the strain with the O9 determinant was suppressed in comparison to that with the O4 determinant.

Parenteral vaccines against enteric bacterial infections are not satisfactory: the vaccines are lacking in efficacy, the immunity they confer is inadequate, and they tend to cause side reactions (22). The vaccines are composed of whole bacteria killed by one or another method. The poor effect and the side reactions are due to two important factors. The endotoxin-containing cell walls are intrinsically noxious. Second, since many different enteric bacteria are found, the question of practicality of vaccine development encompasses not only problems involved in preparation of tens if not hundreds of different vaccines, but also the problem that with each vaccination with crude material one gives a large number of irrelevant antigens and one may well be concerned about antigenic competition (21).

In *Salmonella* there has accumulated evidence that the qualitative constitution of the lipopolysaccharide (a component of the outer membrane containing the O antigen of the bacteria) is of importance for virulence (12, 13). Killed vaccines of bacteria afford specific protection against infection by organisms of homologous O-antigenic type but not against organisms of heterologous type (12, 13). The structure of the polysaccharide part of the lipopolysaccharide with the O-antigenic specificity has been stablished for several *Salmonella* species (6). If key O-antigenic determinants, without endotoxic activity, can be coupled to immunogenic carriers, it might be possible to give protection from infection by many organisms for which no effective prophylaxis is now available. Previous investigations have shown that individual dideoxyhexoses of *Salmonella* were haptenes which functioned as antigenic determinants when coupled to bovine serum albumin (BSA) or egg albumin and injected into goats and rabbits (18, 19). The potential use of these synthetic antigens for eliciting protection was not studied.

The disaccharide methyl-3-*O*-α-tyvelopyranosyl-α-D-mannopyranoside, representative of the O antigen 9 determinant in *Salmonella typhi*, was recently synthesized (2) and found to be 10-fold more active than the methyl-α-D-tyveloside derivative in a precipitation-inhibition assay (unpublished data). This communication described the use of the synthetic disaccharide, covalently linked to BSA as a carrier, for immunization of mice. The mice formed antibody which was effective in a bactericidal assay in vitro against the O9 determinant. A sensitive in vivo test demonstrated that in immunized animals, after challenge with two strains of equal virulence but with different O antigens, the growth of the strain with the O9 determinant was suppressed in comparison with that with an O4 determinant.

MATERIALS AND METHODS

Bacterial strains. *Salmonella enteritidis* Br'203bM (O antigen 9,12) (20) and *S. typhimurium* P173C (O antigen 4,5,12) (11) were obtained from R. J. Roantree, Stanford University. *Salmonella* strains PL5 and PL8 were obtained from P. Lyman, Stanford

University. Strain PL8 is derived from *S. typhimurium* SL4522, a genetically marked subline (*hisC*, *cysI*, *malB*, etc.) of a virulent *S. typhimurium* strain with O antigens 4,5,12 and of type FIRN (8, 10), made *rha*$^+$ by transduction using phage P22.L4 grown on *S. typhimurium* LT2. Strain PL5 is strain SL4522 into which the *rfb* character (O antigens 9,12) from *S. enteritidis* 203 (O9,12) was transferred and subsequently made *mal*$^+$ by transduction with phage P22.L4 grown on *S. typhimurium* LT2. Strains PL5 and PL8 are thus isogenic except for the *rfb* region and the *mal* and *rha* loci. Both strains are of equal virulence and have nearly identical growth rates in normal mouse serum or brain heart infusion broth (P. B. Lyman, Ph.D. thesis, Stanford University, Palo Alto, Calif., 1974).

Disaccharide antigen. The disaccharide derivative [*p*-aminophenyl-3-*O*-(3,6-dideoxy-α-D-*arabino*-hexopyranosyl)-α-D-mannopyranoside] was synthesized as described (2) and coupled to BSA by the formation of the phenylisothiocyanate derivative essentially as described by McBroom et al. (9). There was an average of 4 mol of disaccharide per mol of BSA.

Immunization procedure. Groups of 10 inbred male mice of a strain designated supermouse (15), weighing 15 to 20 g, were injected intraperitoneally with different concentrations of the disaccharide antigen (covalently linked to BSA) suspended in Freund complete adjuvant on days 1, 8, 15, and 22. One group of mice was injected intravenously with the antigen. One group was immunized with BSA suspended in Freund complete adjuvant. Another group was given heat-killed bacteria (PL5), 5×10^7 cells, intraperitoneally.

Hemagglutination test. Experiments were performed with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) by using 25-μliter loops and 2% human erythrocyte suspensions coated with phenol-water-extracted lipopolysaccharides (5). Agglutination was recorded after incubation at room temperature and in the cold for 1 and 18 h, respectively.

Bactericidal assay. The part-rough and complement-sensitive strains *S. enteritidis* Br'203bM O9,12 (20) and *S. typhimurium* P173C O4,5,12 (11) served as test organisms. Approximately 1,500 cells in logarithmic growth phase were mixed with dilutions of mouse sera and guinea pig complement diluted 1:5 in a total volume of 0.6 ml. The mixture was incubated for 60 min at 37 C and plated on nutrient agar plates (Oxoid CM55), and colonies were counted after 18 h of incubation at 37 C. Controls with bacteria and complement and those with only bacteria showed that the complement in the dilution used did not kill the bacteria. The reciprocal of the serum dilution giving 50% killing of the bacteria was determined.

Double-challenge experiment. Immunized and nonimmunized mice were challenged with a mixture of *Salmonella* strains PL5 (O9,12) and PL8 (O4,5,12) (P. B. Lyman, Ph.D thesis); approximately 1,000 to 1,500 bacteria in the logarithmic growth phase in a PL5/PL8 ratio close to 1.0:1.0. The mice were sacrificed on day 3. The liver and spleen were taken out and weighed. Bacterial counts and the ratios of PL8 (O4,5,12) and PL5 (O9,12) in the liver and spleen were determined by making imprints on the surface of indicator plates and by counts of dilutions of liver and spleen homogenates similarly plated. The fermentation markers (maltose and rhamnose) enabled the differential counting of the strains on MacConkey agar plates containing either 1.0% rhamnose or maltose.

RESULTS

The only difference in O-antigenic structure between a *Salmonella* strain with O9,12 specificity (like *S. typhi*) and one with O4,12 specificity (like *S. typhimurium*) is the substitution of one dideoxyhexose, tyvelose (O9 specificity), for another, abequose (O4 specificity) (Fig. 1). The immunizing properties of the synthetic disaccharide methyl 3-*O*-α-D-mannopyranoside when covalently linked to BSA as the carrier were compared with those of heat-killed *Salmonella* PL5 containing the same O9 antigenic determinant.

The humoral antibody response was examined by using a bactericidal assay where *S. enteritidis* Br'203bM (O9,12) and *S. typhimurium* P173C (O4,5,12) served as test organisms. The reciprocal of the serum dilution giving 50% killing of the bacteria was determined. No killing was detected with serum from nonimmunized mice. Seven days after initial immunization, a titer of 128 was detected in pooled serum from mice which had received 25 μg of the synthetic antigen in Freund complete adjuvant. Sera from mice bled on day 39 (17 days after the last immunization) were pooled and assayed (Table 1). The highest bactericidal titer, 10,240, was observed in mice immunized with 25 μg of the synthetic antigen; mice given lower doses of the antigen in adjuvant showed titers ranging from <10 to 2,560. Sera from mice given the antigen without adjuvant, or given BSA with adjuvant, showed no bactericidal titer, i.e., <10. None of the antisera, in dilutions 1:10 or higher, killed *S. typhimurium* strain P173C (O4,5,12), which indicates that the bactericidal antibody response elicited by the synthetic antigen was specific. Sera from mice immunized with heat-killed PL5 bacteria (O9,12) displayed, however, the highest bactericidal antibody titer, 40,960.

Passive hemagglutination using human erythrocytes coated with phenol-water-extracted lipopolysaccharide from PL5 bacteria (O9, 12) showed that the agglutinin titers from mice immunized with the synthetic antigen and bled on days 8, 15, and 39 were low. The highest titer, 20, was observed in pooled sera drawn on day 39 from mice given 25 μg of the synthetic antigen in Freund complete adjuvant. The ag-

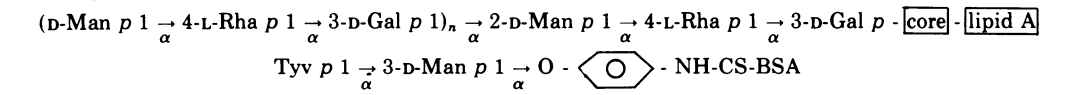

FIG. 1. *Main structural features of the O polysaccharide chain, built by polymerized oligosaccharides, of the lipopolysaccharides of the two Salmonella strains used. In strain PL5 (O9,12), R indicates tyvelose; in strain PL8 (O4,5,12), R indicates 2-O-acetyl abequose. The synthetic antigen used is a phenylisothiocyanato disaccharide with O9 specificity covalently linked to BSA. Abbreviations used: Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; Tyv, tyvelose; n, number of repeating units in O chain, varying from 4 to 30 (6).*

TABLE 1. *Characteristics of the immune response to the synthetic antigen*

Immunizing agent and dose given[a]	Bactericidal titer[b]	Mean bacterial counts in liver-spleen homogenates[c]		Suppression of PL8 in double challenge[d]
		PL8(O4,5,12)	PL5(O9,12)	
25 μg of tyv-man-BSA in FCA i.p.	10,240	2.5×10^6 (3.2×10^4–9.0×10^6)	1.9×10^5 (3.2×10^2–1.0×10^6)	5/7
5 μg of tyv-man-BSA in FCA i.p.	640	3.7×10^6 (1.5×10^6–7.0×10^6)	5.6×10^5 (2.5×10^5–1.0×10^6)	3/5
1 μg of tyv-man-BSA in FCA i.p.	<10	1.3×10^6 (1.6×10^3–6.0×10^6)	8.2×10^5 (1.6×10^3–1.8×10^6	0/6
0.2 μg of tyv-man-BSA in FCA i.p.	2,560	ND	ND	0/7[e]
1.0 μg of tyv-man-BSA i.v.	<10	2.3×10^6 (1.6×10^5–4.3×10^6)	1.0×10^6 (4.3×10^5–2.1×10^6)	0/6
25.0 μg of BSA in FCA i.p.	<10	1.0×10^6 (6.0×10^4–1.5×10^6)	1.3×10^6 (6.7×10^4–1.4×10^6)	0/15
5×10^7 heat-killed PL5(O9,12) bacteria i.p.	40,960	2.6×10^5 (3.6×10^3–6.0×10^5)	1.0×10^4 (4.3×10^2–5.0×10^4)	10/15

[a] The synthetic antigen was suspended in Freund complete adjuvant (FCA) and injected intraperitoneally (i.p.) into mice on days 1, 8, 15, and 22. One group received the synthetic antigen intravenously (i.v.) without adjuvant, one group received BSA with adjuvant, and one group was immunized with heat-killed PL5 bacteria (O-antigen 9,12).

[b] Reciprocal of the dilution of pooled mouse serum causing 50% killing in the presence of guinea pig complement in 60 min at 37 C.

[c] Determined by plating dilutions of liver and spleen homogenates on MacConkey agar plates containing 1% of either maltose or rhamnose. Figures in parentheses represent the range. ND, Not determined: only a qualitative estimation of growth was done by imprints of the livers and spleen on indicator plates.

[d] Mice were challenged intraperitoneally with a mixture of strains PL8 (or 5,12) and PL5 (O9,12) on day 46. The animals were sacrificed on day 49, and the PL8/PL5 ratios were determined; for significant suppression the ratio had to be ≥10.0:1.0.

[e] One of the mice in the group had a ratio of 1:125.

glutinin titers in pooled sera from mice immunized with heat-killed PL5 bacteria were higher: 100 in sera drawn at day 15 and 640 at day 39.

To evaluate whether the immunization with the synthetic antigen had any influence on the in vivo growth of *Salmonella* bacteria with the O9 antigen, a double-challenge experiment was set up. The challenging organisms, strains PL5 (O9,12) and PL8 (O4,5,12), differed only in their O antigen formula and in their ability to ferment maltose and rhamnose. The mean lethal dose for both strains in nonimmunized mice was <20 bacteria when injected intraperitoneally. Immunized mice were infected intraperitoneally with approximately 1,200 bacteria in the logarithmic growth phase on day 46 (24 days after the last immunization). The ratio of PL8 (O4,5,12) to PL5 (O9,12) was 1.20:1. The mice were sacrificed on day 49, 3 days after challenge. Bacterial counts and the ratios of PL8 to PL5 in liver and spleen homogenates were determined. The mean value of the ratio of PL8/PL5 in control mice immunized with 25 μg of BSA in Freund complete adjuvant was 0.8; the mean bacterial count was 10^6 bacteria per g of liver-spleen homogenate for PL8 and 1.3×10^6/g for PL5. The immunization procedure was deemed to give suppression of growth of PL5 (O9,12) when the PL8/PL5 ratio was ≥10.0:1. In mice immunized with 25 μg of the synthetic antigen, the mean ratio was 13.2:1, and the mean bacterial counts were 2.5×10^6 bacteria/g for

PL8 and 1.9×10^5/g for PL5. Five of the seven mice in this group had ratios $\geq 10.0:1$; for three of them the ratios were $\geq 100:1$. In the group given 5 μg of the synthetic antigen, three out of five mice were deemed to have suppression of growth of PL5; the mean bacterial counts were 3.7×10^6 and 5.6×10^5 bacteria/g for PL8 and PL5, respectively. None of the mice given 1.0 μg intraperitoneally in Freund complete adjuvant or 1.0 μg intravenously (without adjuvant) showed suppression of growth of PL5 as judged by the criteria used. Ten of the 15 mice given heat-killed bacteria showed suppression of PL5 growth (ratios of $36:1$ to $>100:1$). The liver and spleen counts in the remaining five mice yielded ratios between $2.2:1$ and $8.0:1$, and the mice were deemed as having no significant suppression of PL5 growth. The mean bacterial counts were 2.6×10^5 and 10^4 bacteria/g for PL8 and PL5, respectively.

DISCUSSION

We have shown that the synthetic disaccharide tyvelosyl $1 \xrightarrow{\alpha} 3$ mannose coupled to BSA elicited a specific humoral antibody response in mice. With the sensitive bactericidal antibody assay, a titer was detected as early as 7 days after initial immunization. A maximal titer of 10,240 was found 17 days after the last of four injections of 25 μg of the synthetic antigen in Freund complete adjuvant given 7 days apart. The bactericidal titer attained after immunization with heat-killed bacteria having the O9 antigenic determinant was higher, 40,960 (Table 1). The synthetic antigen elicited only a low hemagglutinating titer of 20, as compared with a titer of 640 elicited by immunization with the heat-killed bacteria. When the synthetic antigen was used as an immunogen in rabbits, specific antibody production was also detected: bacterial agglutination and the enzyme-linked immunosorbent assay showed specific O9 antibody response in immunized animals (unpublished data). To our knowledge this is the first demonstration that the antibody elicited by immunization with a disaccharide as a hapten covalently linked to a protein carrier displays specific bactericidal activity.

The principal advance in this work, however, is the demonstration that the synthetic antigen used as an immunogen in mice gives rise in vivo to suppression of growth of the bacterium with the O9 antigenic determinant relative to the growth of the bacteria with the O4 determinant (Table 1). The mean ratio for O4/O9 bacteria shifted from $0.8:1$ to $13.2:1$ in mice immunized with 25 μg of the antigen in adjuvant as compared with the group given 25 μg of BSA in adjuvant only. The corresponding mean ratio in nonimmunized mice was $2.6:1$. The mean ratio in mice given heat-killed bacteria was $>50:1$. Suppression of the bacteria with the O9 determinant, judged both as ratio and number of bacteria per gram of liver and spleen homogenate, was thus more effective when heat-killed bacteria were used for immunization as compared with the synthetic antigen. Two facts may account for this. First, immunization with heat-killed bacteria may elicit the formation of bactericidal antibodies directed against other antigens than the O9 determinant; and second, there was only an average of 4 mol of disaccharide per mol of BSA, and 4 is a small number. Theoretically, 57 mol of the disaccharide can be linked per mol of BSA (9). The degree of heterogeneity of coupling is unknown. The possibility that we have been immunizing with a mixture of heavily substituted and perhaps unsubstituted BSA is not excluded. Double-diffusion studies with haptenated and unhaptenated BSA and anti-BSA formed in rabbits showed no evidence of antigenic modification of the BSA.

The suppression in mice immunized with the synthetic antigen roughly correlates with the occurrence of a bactericidal antibody response (Table 1). We do not assert, however, that the suppression in vivo is mediated by the bactericidal antibody. Mouse complement does not bring about bacteriolysis (14). Even if it has been suggested that humoral antibodies against cell-wall components play a role in the resistance to *Salmonella* infections (4, 16), there is mounting evidence that effective resistance is primarily due to cell-mediated immunity (1, 3, 7, 17). Cell-mediated immunity is difficult to demonstrate in the mouse. We have some evidence that guinea pigs immunized with the synthetic disaccharide coupled to BSA in complete Freund adjuvant and challenged with the appropriate lipopolysaccharide express typical delayed skin reactions.

The injections with Freund adjuvant intraperitoneally created a chronic inflammatory response with varying degrees of disruption of the normal peritoneal cavity. Peritoneal adhesions to the viscera were evident in many animals. We chose to delay challenge and sacrifice to about 4 weeks after the last immunizing injection to minimize the effect of the more acute responses. These may be nonspecific and would be controlled by the double-challenge technique. Many experimental immunization procedures arouse non-specific defense mechanisms. Experimental animals may show large increases in average time to death or in mean lethal doses and yet be poorly protected in a specific way. The double-challenge approach

we have used is designed to detect specific suppression of growth of one strain in circumstances where large changes in nonspecific resistance may be expected. The suppression of growth of strain PL5 (O9,12) does not imply that the immunized mice have attained protection as would be measured by tests designed to detect prolongation of life or changes in mean lethal dose.

The limits of the approach remain to be explored. Further work is in order with respect to the following. (i) Can one establish significant resistance to infection as indicated by prolongation of life and reduction of mortality? (ii) Are more suitable carrier molecules sufficiently unaltered to retain their own immunogenicity? In this respect we believe that such widely used immunogens as diphtheria or tetanus toxoids are preeminent candidates.

ACKNOWLEDGMENTS

We express appreciation for the excellent technical assistance of Linda Arnold and Connie Whiteside.

This work was supported by Public Health Service International Fellowship no. F05 TW 1912, the Swedish Medical Research Council (grant no. 40X-656), Public Health Service research grant AI-01768 from the National Institute of Allergy and Infectious Diseases, and by Stanford Research Development Fund.

LITERATURE CITED

1. Blanden, R. V., G. B. Mackaness, and F. M. Collins. 1966. Mechanisms of acquired resistance in mouse typhoid. J. Exp. Med. **124**:585-600.
2. Borén, H., P. J. Garegg, and N. H. Wallin. 1972. Synthesis of methyl 3-*O*-(3,6-dideoxy-α-D-arabinohexopyranosyl)-α-D-mannopyranoside. Acta Chem. Scand. **26**:1082-1086.
3. Collins, F. M. 1969. Effect of specific immune mouse serum on the growth of *Salmonella enteritidis* in nonvaccinated mice challenged by various routes. J. Bacteriol. **97**:667-675.
4. Jenkin, C. R., and D. Rowley. 1963. Basis for immunity to typhoid in mice and the question of "cellular immunity." Bacteriol. Rev. **72**:391-404.
5. Lindberg, A. A., and T. Holme. 1972. Evaluation of some extraction methods for the preparation of bacterial lipopolysaccharides for structural analysis. Acta Pathol. Microbiol. Scand. Sect. B. **80**:751-759.
6. Lüderitz, O., O. Westphal, A.-M. Staub, and H. Nikaido. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides, p. 145-233. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 4. Academic Press Inc., New York.
7. Mackaness, G. B. 1971. Resistance to intracellular infection. J. Infect. Dis. **123**:439-445.
8. MacPhee, D. G., and B. A. D. Stocker. 1969. Suppression of amber and ochre mutants in *Salmonella typhimurium* by a mutant F'-1-gal factor carrying an ochre suppressor gene. J. Bacteriol. **100**:240-246.
9. McBroom, C. R., C. H. Samanen, and I. J. Goldstein. 1972. Carbohydrate antigens: coupling of carbohydrates to proteins by diazonium and phenylisothiocyanate reactions, p. 212-219. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 28B. Academic Press Inc., New York.
10. Morgenroth, A., and J. P. Duguid. 1968. Demonstration of different mutational sites controlling rhamnose fermentation in FIRN and non-FIRN *rha*⁻ strains of *Salmonella typhimurium*: an essay in bacterial archaeology. Genet. Res. **11**:151-169.
11. Ornellas, E. P., R. J. Roantree, and J. P. Steward. 1970. The specificity and importance of humoral antibody in the protection of mice against intraperitoneal challenge with complement-sensitive and complement-resistant Salmonella. J. Infect. Dis. **121**:113-123.
12. Roantree, R. J. 1967. Salmonella O antigens and virulence. Annu. Rev. Microbiol. **21**:443-466.
13. Roantree, R. J. 1971. The relationships of lipopolysaccharide structure to bacterial virulence, p. 1-37. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 5. Academic Press Inc., New York.
14. Rosenberg, L. T. 1965. Complement. Annu. Rev. Microbiol. **19**:285-300.
15. Rosenberg, L. T., and D. K. Tachibana. 1969. On mouse complement: genetic variants. J. Immunol. **103**:1143-1148.
16. Rowley, G., J. Auzins, and C. R. Jenkin. 1968. Further studies regarding the question of cellular immunity in mouse typhoid. Aust. J. Exp. Biol. Med. Sci. **46**:447-463.
17. Saito, K., and S. Mitsuhashi. 1965. Experimental salmonellosis. VI. In vitro transfer of cellular immunity of mouse mononuclear phagocytes. J. Bacteriol. **90**:629-634.
18. Staub, A. M., S. Stirm, L. LeMinor, O. Lüderitz, and O. Westphal. 1966. Essai de production d'anticorps anti-enterobacteriaceae au moyen d'antigènes artificiels portant des 3,6-didésoxyhexosides comme groupes determinants. Ann. Inst. Pasteur (Paris) **111**(Suppl. 5): 47-58.
19. Stellner, K., O. Lüderitz, O. Westphal, A.-M. Staub, B. Leluc, C. Coynault, and L. LeMinor. 1972. Immunochemical studies on "Salmonella." Serological specificity of 2-O-acetyl-abequose and the production in rabbits of anti-5 antibodies with artificial antigens. Ann. Inst. Pasteur (Paris) **123**:43-54.
20. Steward, J. P., L. R. Collis, and R. J. Roantree. 1966. Effects of immunization of guinea pigs lacking bactericidal antibody against *Salmonella enteritidis*. J. Immunol. **97**:224-230.
21. Taussig, M. J. 1970. Antigenic competition. Curr. Top. Microbiol. Immunol. **60**:125-174.
22. World Health Organization. 1972. Oral enteric bacterial vaccines. WHO Technical Report Series, No. 500. Geneva, Switzerland.