Serological Relatedness of Mouse-Virulent *Yersinia enterocolitica*

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An antiserum (WA-SAA) was produced which agglutinated specifically with mouse-virulent but not with avirulent strains of *Yersinia enterocolitica*. Expression of the antigenic determinant(s) reacting with WA-SAA was temperature dependent; for growth temperatures of 20 to 40°C, agglutination titers were lowest for cultures grown at 20°C and highest for cultures grown at 35 to 40°C. Addition of Ca^{2+} (2.5 to 10 mM) to the growth medium had little effect on the agglutination titer, and gel diffusion studies with monospecific anti-V serum indicated that V antigen was not likely to be the determinant reacting with WA-SAA. Immunohistological studies of Peyer's patches of mice infected with *Y. enterocolitica* WA revealed that the antigenic determinant(s) reacting with WA-SAA was expressed in vivo. The strong correlation of agglutination titer with mouse virulence and the expression in vivo of the antigenic determinant(s) reacting with WA-SAA suggest that the antigen(s) may be associated with the pathogenicity of *Y. enterocolitica*.

Yersinia enterocolitica is a widely distributed organism which may be isolated from a variety of foods. Examples include milk (17, 30), fish (20), porcine tongues (12), chicken (23), lamb (15), beef (15), oysters (1, 25), shrimp (25), crab (25), and vegetables used in salads (1). Although the organism is often present in foods and the environment, a large number of the strains isolated from these sources are apparently avirulent (21, 28, 29). Hence, it would be useful to identify characteristics common among virulent *Y. enterocolitica* that may be used as determinants for developing methods to differentiate virulent from avirulent strains.

Several established or presumed virulence determinants have been associated with *Yersinia pestis*. Included are the ability to produce V and W antigens, capsular or fraction 1 antigen, pesticin, coagulase, and fibrinolysin, and the capacity to absorb hemin (5). Carter et al. (11) have shown that mouse-virulent *Y. enterocolitica* WA produces V and W antigens that are immunologically identical to the V and W antigens of *Y. pestis*. These are the only virulence determinants known to be common to these two species; however, it is not known whether V and W antigens are common among all strains of mouse-virulent *Y. enterocolitica*. Interestingly, although not a determinant of virulence, susceptibility to pesticin, a bacteriocin produced by wild-type strains of *Yersinia pestis* (4), is also a characteristic common among strains of *Y. enterocolitica* that are lethal to mice (16).

We report here a serological test that identi-

fied virulent strains of *Y. enterocolitica* under growth conditions in which plague virulence V antigen was not detected. The antigenic determinant(s) reacting with WA-specific absorbed antiserum (WA-SAA), termed WA-SAA Ag, was expressed equally well in the presence of 2.5 to 10 mM calcium and could be demonstrated in vivo in the Peyer's patches of infected mice. The antigenic determinant(s) appears to be a new virulence factor(s) not reported previously.

MATERIAL AND METHODS

Organisms. Cultures used for this study are shown in Table 1. Each isolate was individually stored frozen at −20°C as thick suspensions in 2 ml of 30% glycerol–1% peptone until used.

Bacterial antigen. Live *Y. enterocolitica* WA cells were used for immunization. Cultures were prepared by transferring one loopful of culture stock into each of two tubes containing 10 ml of Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) and incubating for 18 h at 37°C. After incubation, cells were concentrated by centrifugation (2,000 × *g*, 20 min) and washed and suspended in 0.01 M phosphate-buffered saline, pH 7.2 (PBS). A fresh culture of cells was prepared for each day that the animals were immunized.

Production of antiserum. Three white New Zealand rabbits weighing 2.5 to 3.0 kg were used for immunizations. The animals were given a total of 19 0.1-ml intravenous injections of the bacterial antigen as described by Barber and Eylan (2). Doses ranging from 1.5×10^9 to 3.0×10^9 viable cells were given at 2- to 3-day intervals. After 11 injections, the animals were allowed to rest for 12 days and were then given 8 additional injections. Rabbits were bled 11 days after

the last injection. The resulting serum is referred to as anti-WA serum. Only anti-WA serum from one rabbit, i.e., that antiserum which had the highest agglutination titer with strain WA and the lowest titer with strain WA-ETBR, an attenuated, noninvasive derivative of *Y. enterocolitica* WA (13), was used to prepare WA-SAA.

Absorption of antiserum. Anti-WA serum was absorbed extensively with strain WA-ETBR. Strain WA-ETBR was grown for 18 h at 37°C in 100 ml of TSB contained in each of 40 500-ml Erlenmeyer flasks. Cells were harvested by centrifugation, washed twice with PBS, and divided into four equal fractions, each containing approximately 1 ml of packed cells. Cells of each fraction were suspended in 100 ml of PBS, with two fractions being autoclaved (121°C, 1 h) and the remaining two being treated with 0.3% (vol/vol) Formalin at room temperature for 15 min. Cells were centrifuged, washed with PBS, and held at 4°C.

Anti-WA serum (10 ml) was heated at 56°C for 30 min, added to 1.0 ml of packed, heat-treated cells of strain WA-ETBR, and mixed gently at 37°C for 30 min. The serum was recovered by centrifugation (10,000 × g, 20 min). Further absorption was done by one additional incubation with heat-killed cells and two more incubations with formalinized cells of strain WA-ETBR as described previously. This exhaustively absorbed antiserum is referred to as WA-SAA.

Titration of WA-SAA. The antibody agglutination test was done with 18-h cultures of *Y. enterocolitica* grown at 37°C in TSB. Cells were harvested by centrifugation and washed and adjusted to an optical density of 2.0 at 520 nm with PBS. Twofold dilutions of WA-SAA were done in microtitration plates (Titertek; Flow Laboratories, Inc., McLean, Va.) with V-shaped wells containing 50 µl of PBS–0.02% bovine serum albumin. Bacterial culture (50 µl) was added to each well of diluted antiserum, and plates were incubated at 37°C for 1 h and held at 4°C for 24 h. The highest dilution of antiserum showing agglutination was taken as the titer. For each culture, wells containing PBS-bovine serum albumin only, PBS-bovine serum albumin and bacterial culture, and PBS-bovine serum albumin and WA-SAA served as controls to show that nonspecific agglutination was not occurring.

Purification of WA-SAA IgG. WA-SAA immunoglobulin G (IgG) was precipitated by slowly adding saturated ammonium sulfate to equal volumes of double-distilled water and WA-SAA (5 ml each) to obtain 33% saturation of $(NH_4)_2SO_4$, allowing the solution to stand overnight at 4°C and precipitating the globulin fraction by centrifugation (12,000 × g, 20 min). The precipitate was dissolved in 5 ml of 0.02 M Tris-hydrochloride buffer, pH 8.0, containing 0.5 M NaCl (Tris buffer) and dialyzed overnight at 4°C against three to four changes (150- to 200-ml volumes per change) of Tris buffer. Precipitate remaining after dialysis was removed by centrifugation (12,000 × g, 10 min), and the supernatant fluid was fractionated by gel filtration (Sephacryl S-300; Pharmacia Fine Chemicals, Uppsala, Sweden) with Tris buffer as the eluent. IgG fractions constituting the second major peak (optical density at 280 nm) were pooled, concentrated by ultrafiltration with 43-mm diameter Amicon XM100A filters (Amicon Corp., Lexington, Mass.), suspended in 0.15 M NaCl, and frozen at −20°C in 1-ml portions.

Virulence testing. Virulence was determined by

expression of diarrhea or death in orally dosed mice as described previously by Doyle et al. (12) and Schiemann et al. (29). Mice were examined twice daily for death and at 2, 5, 9, and 14 days after challenge for diarrhea. Symptoms of diarrhea were assessed by placing the mice in individual wire cages over filter paper for 2 h. Collected feces were examined for evidence of diarrhea by comparison with normal feces. Diarrheal specimens had a softened or viscous, liquid consistency and were generally light brown.

Assay for in vivo expression of WA-SAA Ag reacting with WA-SAA. Each of three groups of five mice was perorally challenged with virulent strain WA, avirulent strain WA-ETBR, or sterile water as described by Doyle et al. (12). One animal from each group was sacrificed on days 0, 2, 3, 4, and 5 after challenge, and three or four of the most distal ileal Peyer's patches and adjacent ileal tissue were removed. Tissue was sectioned in 3- to 5-mm lengths, examined for gross abnormalities, and immersed in 4% paraformaldehyde containing 0.075 M lysine and 0.0375 M phosphate buffer, pH 6.2 (4°C). Tissues were washed, embedded, and mounted by the method of Isobe et al. (18). Modifications of this procedure (18) were used to immunohistochemically stain the tissue. After mounting, tissue-embedded slides were washed with cold PBS (15 min each, three changes), immersed for 15 min in 0.5% periodic acid (to inactivate endogenous peroxidase activity), washed with PBS, immersed in 0.003 M sodium borohydride (30 min), and washed with PBS. The tissue was then covered with 5% normal goat serum in PBS for 30 min and washed with PBS. Tissue was flooded with WA-SAA IgG (0.05 mg/ml), washed with PBS after 45 min, and covered with goat anti-rabbit peroxidase-labeled IgG (0.03 mg/ml; Miles Laboratories, Inc., Elkhart, Ind.). After 45 min, the tissue was washed in PBS and treated with Karnovsky diaminobenzidine solution (10 min). Slides were washed, mounted, and examined by light microscopy.

As a negative control, tissue from mice challenged with *Y. enterocolitica* WA was treated as above except that no WA-SAA IgG was added. No peroxidase activity could be detected. An additional control confirmed that cells of strain WA-ETBR ($10^9/3$ cm^2) grown in TSB did not react visibly at a magnification of ×48 with the WA-SAA–goat anti-rabbit IgG peroxidase complex, whereas a comparable concentration of cells of strain WA reacted strongly.

Tissue sections from each treatment were also counterstained with hematoxylin and eosin and examined microscopically for abnormalities of tissue structure and changes in cellular appearance.

RESULTS AND DISCUSSION

Reaction of WA-SAA with different strains of *Y. enterocolitica*. A total of 39 isolates were tested for their ability to agglutinate with WA-SAA (Table 1). Included were several strains of serotype O:8, which is the serotype predominantly associated with yersiniosis in humans in the United States (3, 33); serotype O:3, which is the serotype predominantly associated with yersiniosis in Canada (31) and Japan (35); and serotype O:9, which together with serotype O:3

TABLE 1. Reaction of *Y. enterocolitica* WA-SAA with different strains of *Y. enterocolitica*

Strain	Serotype	Origin	Mouse virulence		Titer[a]	Reference
			Diarrhea	Death		
WA	O:8	Human blood	+	+	15,360	13
WA-ETBR	O:8	Plasmid-cured derivative of WA[b]	−	−	<2	13
Y7P	O:8	Human	+	+	15,360	13
Y7N	O:8	Plasmid-cured derivative of Y7P[b]	−	−	<2	13
CDC A2635	O:8	Chocolate milk	+	+	15,360	—[d]
FRI-A2635N	O:8	Plasmid-cured derivative of CDC A2635[c]	−	−	<2	—[e]
FRI-YE1	O:8	Porcine tongue	−	+	7,680	12
FRI-YE3	O:8	Porcine tongue	+	+	1,920	12
FRI-YE5	O:8	Porcine tongue	−	+	30,720	12
FRI-YE9	O:8	Porcine tongue	ND[f]	+	15,360	12
FRI-YE10	O:8	Porcine tongue	+	+	15,360	12
FRI-YE13	O:8	Porcine tongue	+	+	7,680	12
E661	O:8	Human stool	+	+	15,360	29
700	O:3	Human	+	−	1,920	—[g]
C122-76	O:3	Human stool	+	−	960	9
C108-76	O:3	Human abscess	+	−	7,680	9
IP134	O:3	Human mesenteric node	−	−	<2	—[h]
FRI-YE11	O:3	Porcine tongue	+	−	1,920	12
FRI-YE16	O:3	Porcine tongue	+	−	1,920	12
6809	O:3	Human stool	−	−	<2	27
E675	O:3	Human stool	+	−	960	29
E705	O:9	Human stool	+	−	7,680	29
IP383	O:9	Human stool	+	−	1,920	—[h]
E701	O:4,32	Human stool	+	+	1,920	29
E654	O:5,27	Human stool	+	−	960	29
E657	O:5,27	Human stool	+	−	960	29
E659	O:5,27	Human stool	−	−	<2	29
E736	O:21	Human stool	+	+	7,680	29
84	O:5	Porcine tonsils	−	−	<2	14
14011	O:5	Human stool	−	−	<2	34
15750	O:5	Human stool	−	−	<2	34
FRI-YE12	O:6,30	Porcine tongue	−	−	<2	12
FRI-YE14	O:6,30	Porcine tongue	−	−	<2	12
FRI-YE15	O:6,30	Porcine tongue	−	−	<2	12
29828	O:7,8	Human stool	−	−	<2	34
FRI-YE8	O:13,7	Porcine tongue	−	−	<2	12
FRI-YE2	O:18	Porcine tongue	−	−	<2	12
11051A	O:28	Shrew	−	−	<2	19
FRI-YE6	O:46	Porcine tongue	−	−	<2	12

[a] Titers are expressed as reciprocals.
[b] Ethidium bromide cured.
[c] Spontaneously cured.
[d] I. J. Mehlman, Food and Drug Administration, Washington, D.C.
[e] M. P. Doyle, Food Research Institute, Madison, Wis.
[f] ND, Not determined.
[g] C. H. Pai, McGill University-Montreal Children's Hospital Research Institute, Montreal, Quebec, Canada.
[h] G. Wauters, Cliniques Universitaries St. Luc, Brussels, Belgium.

is one of the predominant human serotypes in Europe (22). Also tested were other virulent but less frequently isolated serotypes, including O:5,27, which is the second most commonly isolated serotype associated with human infections in Canada (29) and is occasionally associated with human infection in other countries (24, 32), and serotypes O:4,32 and O:21.

Agglutination titers ranging from 1:960 to 1:15,360 were found among all of the virulent strains of *Y. enterocolitica* that caused either diarrhea or death in mice. Virulent strains that were cured of their 42-megadalton virulence plasmid (WA-ETBR, Y7N, and FRI-A2635) (13, 36) produced neither diarrhea nor death in mice and were also negative (<1:2) in agglutination

TABLE 2. Reaction of WA-SAA with different strains of Y. enterocolitica serotype O:8 grown at different temperatures

Temp (°C)	Titer[a]		
	FRI-YE1	WA	WA-ETBR
20	60	60	ND[b]
25	120	240	ND
28	240	ND	ND
32	1,920	ND	ND
35	3,840	ND	ND
37	3,840	15,360	<2
40	7,680	ND	ND

[a] Titers are expressed as reciprocals.
[b] ND, Not determined.

reactivities. Similarly, several other strains, including three each of serotypes O:5 and O:6,30 and one each of O:7,8, O:13,7, O:18, O:28 and O:46, were avirulent for mice and did not agglutinate (titer, <1:2) with WA-SAA. WA-SAA Ag thus appeared to correlate strongly with virulence of Y. enterocolitica.

The agglutination titer, however, did not appear to correlate with the degree of virulence in mice as exemplified by strains with low agglutination titers which were highly virulent and caused death in mice.

Growth temperature and expression of WA-SAA Ag. A study was done to determine whether the production of WA-SAA Ag is temperature dependent. Two strains of mouse-virulent serotype O:8 were grown in TSB at temperatures ranging from 20 to 40°C and were tested for agglutination as described above (Table 2).

Production of WA-SAA Ag was temperature dependent for both strains, with relatively small amounts (titers, 1:60) being produced at 20°C and large quantities being produced at 35 to 40°C. Titers of 1:3,840 to 1:7,680 were obtained for strain FRI-YE1 at 35 to 40°C, and a titer of 1:15,360 for strain WA at 37°C. As expected, mouse-avirulent strain WA-ETBR did not produce detectable WA-SAA Ag, having a titer of <1:2 when grown at 37°C.

Effect of calcium on production of WA-SAA Ag. A major determinant of virulence of Y. pestis is its ability to produce V and W antigens (6–8). Both V and W antigens are always produced together, and their production is Ca^{2+} dependent, being produced optimally under aeration at 37°C in an enriched medium that lacks Ca^{2+} but contains at least 20 mM Mg^{2+} (4). Carter et al. (11) have shown that these antigens are also produced by Y. enterocolitica WA at 37°C in a Ca^{2+}-deficient medium containing 20 mM Mg^{2+}; however, it was not reported whether the presence of calcium represses the production of V and W antigens.

Since V and W antigens are the only known virulence determinants of Y. enterocolitica, we thought it would be useful to examine the possible relationship of V and W antigens to WA-SAA Ag by assessing the effect of calcium on the production of WA-SAA Ag. We found that, in contrast to the effect of calcium on the production of V and W antigens by Y. pestis, WA-SAA Ag was suppressed when calcium was sequestered in the medium and that added calcium (2.5 to 10 mM) had little effect on WA-SAA Ag production (Table 3). These results suggest that WA-SAA Ag is not likely to be V or W antigens, either separately or in combination.

To further test whether V antigen is produced under our cultural conditions (TSB, 37°C, 18 h), the cytosol of strain WA was tested against monospecific anti-V serum (obtained from R. R. Brubaker, Michigan State University) and WA-SAA by the gel diffusion technique described by Carter et al. (11). The cytosol was obtained from a sonicated cell extract centrifuged at 48,000 × g for 1 h and adjusted to 1.4 mg of protein per ml. V antigen was not detected in TSB-grown strain WA, which gave high agglutination titers with WA-SAA (Fig. 1). V antigen was only detected when the same strain was grown under the same conditions but in brain heart infusion broth (data not shown), probably because TSB contains enough Ca^{2+} to repress production of V antigen, whereas brain heart infusion broth contains rela-

TABLE 3. Reaction of WA-SAA with Y. enterocolitica WA and WA-ETBR grown at 37°C in TSB with or without added calcium or sodium oxalate and magnesium chloride

Growth medium	Titer[a]			
	WA			WA-ETBR, trials 1, 2, and 3
	Trial 1	Trial 2	Trial 3	
TSB	3,840	1,920	3,840	<2
TSB + oxalate + Mg^{2+}[b]	480	ND[c]	ND	<2
TSB + 2.5 mM $CaCl_2$	3,840	1,920	3,840	<2
TSB + 5.0 mM $CaCl_2$	1,920	960	7,680	<2
TSB + 10 mM $CaCl_2$	ND	3,840	1,920	<2

[a] Titers are expressed as reciprocals.
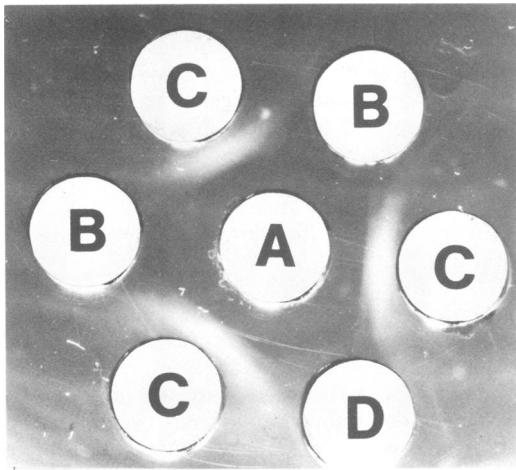[b] Sodium oxalate (20 mM) and 20 mM $MgCl_2 \cdot 6H_2O$.
[c] ND, Not determined.

FIG. 1. Gel immunodiffusion reaction of cytosol extract of TSB-grown strain WA (A) with anti-V serum (B) and WA-SAA (C). Well D is a control containing PBS buffer only.

tively little Ca^{2+}. In both cases, the agglutination titer with WA-SAA was comparable. These observations suggest that antigenic determinants other than V antigen are the primary reactants with WA-SAA.

In studying plasmid-associated determinants of *Y. enterocolitica* pathogenesis, Portnoy et al. (26) found that a minimum of three additional polypeptides were present in outer-membrane sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles derived from gerbil-virulent plasmid-bearing cells grown to stationary phase in brain heart infusion broth at 37°C and that they were not present in the same strain grown at 25°C or in an avirulent plasmid-cured counterpart grown at 25 or 37°C. It was also shown that the addition of 2.5 mM Ca^{2+} to the growth medium completely repressed expression of these plasmid-associated polypeptides. Since the addition of calcium had a repressive effect on the production of these outer-membrane polypeptides and had very little effect on the expression of WA-SAA Ag, it would appear that the polypeptides observed by Portnoy et al. (26) are not the major antigens reacting with WA-SAA. Studies are in progress to identify the antigenic determinant(s) reacting with WA-SAA.

In vivo expression of antigen(s) reacting with WA-SAA. *Y. enterocolitica* WA produced readi-
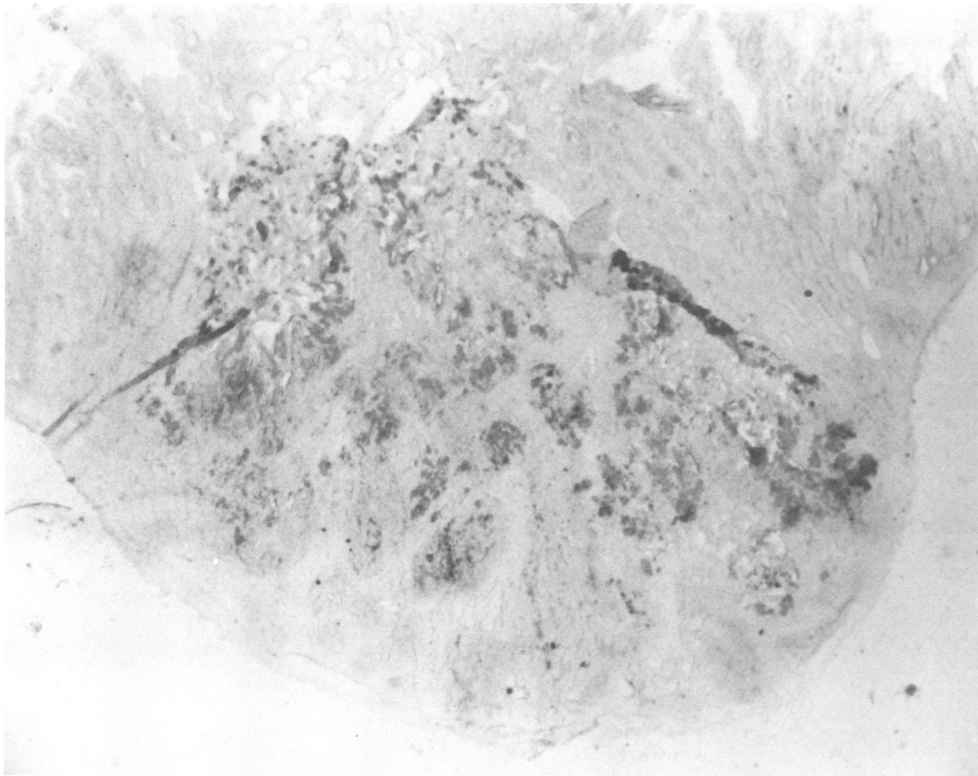

FIG. 2. Ileal Peyer's patch and associated intestinal tissue of mouse 5 days after peroral challenge with *Y. enterocolitica* WA ($\times$48). Tissue was treated with WA-SAA and then with peroxidase-conjugated anti-rabbit IgG and Karnovsky diaminobenzidine solution. Dark-staining loci within the tissue represent concentrated pockets of strain WA in which the organisms are expressing WA-SAA Ag.
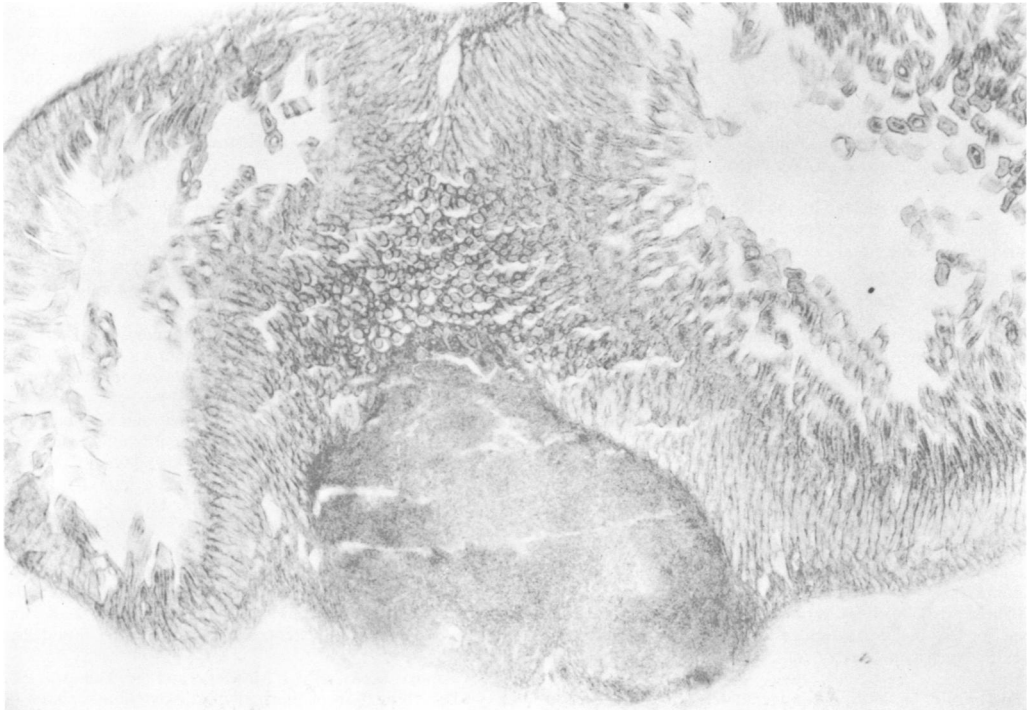
FIG. 3. Ileal Peyer's patch and associated intestinal tissue of mouse 5 days after peroral challenge with *Y. enterocolitica* WA-ETBR (×48). Tissue was treated as described in the legend to Fig. 1. Peyer's patch appears normal and contains no dark-staining WA-SAA Ag.

ly visible, severe, and marked changes in the Peyer's patches of the distal ileum of mice as early as 2 days after challenge. By day 5, the intestinal diameter in the proximity of the Peyer's patches increased two- to threefold; Peyer's patches exuded polymorphonuclear leukocytes, small and medium lymphocytes, and macrophages; there also was evidence of external hemorrhaging in the vasculature of the Peyer's patches. Similar observations were reported by Carter (10). Ileal Peyer's patches and intestinal tissue from mice challenged with *Y. enterocolitica* WA-ETBR evidenced none of these abnormalities through day 5 and were histologically indistinguishable from tissues of nonchallenged control mice.

Figure 1 illustrates the response of a Peyer's patch and surrounding ileal tissue of a mouse 5 days after challenge with strain WA. For comparison purposes, similar tissue of a mouse challenged with strain WA-ETBR is shown in Fig. 2. The Peyer's patch of the mouse infected with strain WA was more than two times larger than that of the equivalent infected with strain WA-ETBR. Furthermore, cells of strain WA infecting the Peyer's patch reacted specifically with WA-SAA as evidenced by staining with an indirect immunoperoxidase technique. This demonstrates that the antigenic determinant(s) of virulent *Y. enterocolitica* which react with WA-SAA is produced by the organism in vivo and suggests that such antigen(s) may be associated with the virulence potential of the organism.

Conclusions. Data indicate that there is a strong correlation between virulence of *Y. enterocolitica* and the possession of an antigen(s) that reacts with WA-SAA. Although the titers of virulent strains reacting with WA-SAA were not identical, all mouse-virulent strains did agglutinate with the antiserum, whereas none of the avirulent isolates reacted. These data support the concept that virulent *Y. enterocolitica* possesses a common antigen unique to virulent strains. Furthermore, this antigen is expressed by the virulent strain in the infected host, suggesting that it may be a virulence factor associated with the pathogenicity of the organism.

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