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Monoclonal Antibodies Delineate Multiple Epitopes on the O Antigens of *Salmonella typhi* Lipopolysaccharide

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Fifteen monoclonal antibodies (MAbs) directed against *Salmonella typhi* were produced and characterized. The specificities of the antibodies were determined by their binding patterns in an enzyme immunoassay, with a panel of lipopolysaccharides isolated from different bacteria. Seven MAbs reacted with *S. typhi*, *Salmonella enteritidis*, and *Salmonella dublin* (all belonging to serogroup D). One MAb also reacted with *Salmonella paratyphi* A and *S. paratyphi* B. Five MAbs reacted with *S. typhi*, *S. enteritidis*, *S. dublin*, and *S. paratyphi* B. Two MAbs did not bind to any lipopolysaccharide but showed reactivity with bacterial sonic extracts isolated from *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, *Escherichia coli*, and *Shigella sonnei*. These antibodies would be helpful in studying the complexity of antigenic determinants expressed by *S. typhi* and the nature of the antibody response during typhoid and paratyphoid fevers and also in the diagnosis of the disease.

Typhoid fever continues to be one of the major public health problems in many countries of the world. The disease, reported as far back as 1659, is still not very well understood in terms of its pathogenesis. Although a heat-killed vaccine against typhoid has been in use for a long time, it confers only short-term immunity and does not prevent relapses (2). Antigens implicit in protective immunity are inadequately characterized. Monoclonal antibodies (MAbs) to *Salmonella* lipopolysaccharides (LPS) and O polysaccharides are reported to protect mice against challenge with virulent *Salmonella typhimurium* (1). LPS and O antigens are, however, complex entities, and one should expect multiple determinants on these antigens. Although hybrid cell clones have been generated previously against *S. typhimurium*, there has been only one report of hybrid cell clones having been generated against *Salmonella typhi* (7). The present communication describes 15 stabilized hybrid cell clones secreting MAbs of diverse specificity. Besides their utility in the delineation of antigenic determinants, these MAbs would be helpful in the early diagnosis of typhoid fever because of their ability to detect the main causative factor of pyrexia, the endotoxin.

MATERIALS AND METHODS

Bacteria. *S. typhi* used for the immunization of mice was an isolate from a patient. It was characterized as serotype O-901. Bacterial strains of *Salmonella paratyphi* A, *S. paratyphi* B, *Escherichia coli*, *Shigella sonnei*, and *Pseudomonas aeruginosa* were obtained from the Department of Microbiology, All India Institute of Medical Sciences, New Delhi. These microorganisms were well defined by culture characteristics and biochemical properties. The bacteria were grown on nutrient agar (Difco Laboratories, Detroit, Mich.) overnight at 37°C, harvested, and heated at 60°C for 30 min. *Salmonella dublin* was obtained from Central Research Institute, Kasauli, India.

Antigen preparation. LPS were isolated and purified from different bacteria essentially by the phenol-water extraction method of Westphal and Jann (12). LPS from *Salmonella enteritidis* was obtained from Difco Laboratories.

Bacterial sonic extracts were prepared by exposing the bacterial suspensions to ultrasonic waves in a Sonifier (model B-30, Branson Sonic Power Co., Danbury, Conn.) at a 50% duty cycle for 3 min. The sonic extracts were centrifuged at 12,000 × *g*, and supernatants were used as a source of antigens.

Production of mouse hybrid cell clones. BALB/c by J.NII mice bred at the Institute Animal Facility were immunized subcutaneously with 100 μg of the sonic extract emulsified with incomplete Freund adjuvant. After 4 weeks a booster injection of 50 μg of sonic extract in incomplete Freund adjuvant was given intraperitoneally. Animals having good antibody titers against *S. typhi*, as tested by a solid-phase enzyme immunoassay (EIA), were given 10 μg of sonic extract in saline intravenously on 3 consecutive days before fusion. In another protocol, mice were immunized intraperitoneally with 2×10^7 heat-killed *S. typhi* bacteria emulsified with incomplete Freund adjuvant and boosted 4 weeks later intraperitoneally with 1×10^7 bacteria in incomplete Freund adjuvant. After 1 to 2 months a final injection of 2×10^7 bacteria was given intravenously.

Hybrid cell clones were developed by fusing splenocytes with SP2/O-Ag 1.4 mouse myeloma cells as described elsewhere (3). Hybrid cells from positive wells were cloned by limiting dilution and subcloned repeatedly to obtain stable cell lines secreting antibodies. Hybrid cells were grown in the peritoneal cavity of Pristane (Aldrich Chemical Co., Milwaukee, Wis.)-primed BALB/c mice as ascites. Ascites fluid tapped from the peritoneal cavity was made cell-free by centrifugation at 800 × *g* for 15 min at 4°C. It was heat inactivated at 56°C for 30 min and centrifuged at 15,000 × *g* to remove debris.

Analysis of MAbs. MAbs were screened for their reactivity by an enzyme-linked immunosorbent assay. Briefly, 96-well polystyrene microtitration plates (Flow Laboratories, England) were coated with 0.1 ml of either LPS (20 μg/ml in 50 mM carbonate buffer, pH 9.6) or bacterial sonic extract antigen (20 μg/ml dissolved in phosphate-buffered saline PBS; 50 mM phosphate, 0.15 M NaCl [pH 7.4]). Antigen coating was done by incubating the plates overnight at 37°C. In another protocol, plates were coated with whole bacteria (15×10^6 per well in carbonate buffer) and dried at 37°C. After coating, plates were washed with PBS-Tween (PBS containing 0.05% Tween 20) and nonspecific binding sites

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TABLE 1. Fine specificity analysis of anti-*S. typhi* MAbs as determined by their reactivity[a] with a panel of LPS

MAb	A_{490} with following LPS:							Antigen used for immunization	Isotype
	S. typhi (9, 12)[b]	*S. enteritidis* (1, 9, 12)	*S. dublin* (1, 9, 12)	*S. paratyphi* A (1, 2, 12)	*S. paratyphi* B (1, 4, 5, 12)	*E. coli*	*S. sonnei*		
P3B5A10	2.625	2.571	2.606	0.089	0.107	0.025	0	*S. typhi* sonic extract	IgG3(κ)
P3C4B2	2.207	1.477	1.742	0	0	0	0		IgG1(κ)
P1C4C9	2.467	2.436	2.649	0	0	0	0		IgG2a(κ)
P1C3H6	2.441	2.373	2.417	0	0	0.041	0		IgG3(κ)
P1C3A5	1.378	0.637	0.845	0	0	0.019	0		IgG3(κ)
P3D6G5	2.459	1.838	2.433	0	0	0.022	0		IgG3(κ)
P3B5G5	2.613	1.834	2.631	0	0.098	0.089	0.086		IgG3(κ)
P3B2F7	2.194	1.537	2.526	2.699	2.568	0.015	0		IgG2a(κ)
P5C5B10	2.719	2.739	2.577	0	0.872	0	0	*S. typhi* intact bacteria	IgG1(κ)
P5C6D1	2.271	2.537	2.276	0	0.996	0	0		IgG1(κ)
P6C3G9	2.510	2.397	2.270	0	0.932	0.014	0		IgG1(κ)
P5C5B2	2.593	2.594	2.642	0.062	2.233	0	0		IgG1(κ)
P5C6E3	2.694	2.487	2.343	0	1.092	0	0		IgG1(κ)
P1A2B10	0.027	0.006	0.099	0	0	0.025	0	*S. typhi* sonic extract	IgG2a(κ)
P1A2A11	0	0.001	0	0	0	0.043	0		IgG2a(κ)

[a] The assay is described in Materials and Methods. Values expressed as A_{490} are the averages of three sets of experiments run in duplicate. The mean absorbance value for various controls was 0.07 ± 0.027.
[b] Numbers within parentheses are the O antigens expressed.

were blocked by adding 100 μl of 2% bovine serum albumin in PBS per well and incubating the plates at 37°C for 1 h. In the case of whole-cell enzyme-linked immunosorbent assay, blocking was done without prior washing. EIA plates were subsequently washed with PBS-Tween and incubated with 100 μl of the tissue culture supernatant. Control wells had either PBS-bovine serum albumin, tissue culture supernatant from SP2/O-Ag 1.4 myeloma cells, or supernatant from unrelated mouse hybrid cell clones. Plates were incubated for 2 h at 37°C and subsequently washed with PBS-Tween. Wells were filled with 100 μl of sheep anti-mouse immunoglobulin coupled to horseradish peroxidase at the appropriate dilution and incubated for 1 h at 37°C. After the plates were washed with PBS-Tween, enzyme activity was determined by adding 100 μl of freshly prepared substrate solution (0.5 mg of *o*-phenylenediamine dissolved in 1 ml of 50 mM citrate buffer, pH 5.6, containing 0.03% hydrogen peroxide [H_2O_2]). The reaction was stopped by adding 50 μl of 5 N H_2SO_4, and the A_{490} was read in an enzyme-linked immunosorbent assay reader (Bio-tek Instruments, Inc., Burlington, Vt.). An A_{490} of higher than the mean plus 3 standard deviations of the control value was taken as positive. MAbs with a mean A_{490} of less than 0.5 but more than the mean plus 3 standard deviations of the control value were scored as low reactive ones.

Heavy- and light-chain specificity of the MAbs was determined by a solid-phase enzyme-linked immunosorbent assay, using alkaline phosphatase-tagged goat anti-mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgG3, μ, λ, and κ chains, kindly supplied by John Kearney, Basel Institute of Immunology, Basel, Switzerland.

Reactivity of the MAbs was also studied by Western blot (immunoblot) analysis. LPS and bacterial sonic extracts were electrophoresed in a 15% sodium dodecyl sulfate–polyacrylamide gel under reducing conditions according to the procedure described by Laemmli (6). The separated components were then transferred to nitrocellulose by the Western blot method described by Towbin et al. (10). Nitrocellulose strips were saturated with 10% bovine serum albumin in PBS, washed with PBS-Tween, and incubated with the appropriate dilution of ascites fluid followed by sheep anti-mouse horseradish peroxidase. Enzyme activity on nitrocellulose paper was revealed by developing the color

TABLE 2. Reactivity[a] pattern of MAbs P1A2B10 and P1A2A11 with different bacterial sonic extracts

Bacterial species	A_{490}	
	P1A2B10	P1A2A11
S. typhi	1.151	1.239
S. paratyphi A	1.385	1.456
S. paratyphi B	1.036	1.011
E. coli	1.438	1.515
S. sonnei	0.952	0.863
P. aeruginosa	0.055	0.031

[a] The assay is described in Materials and Methods. Values represent averages of three sets of experiments run in duplicate. The mean absorbance value for various controls was 0.07 ± 0.027.

TABLE 3. Reactivity[a] pattern of anti-*S. typhi* MAbs with a panel of closely related bacteria[b]

MAb	A_{490} with following bacteria:					
	S. typhi (9, 12)[c]	*S. dublin* (1, 9, 12)	*S. paratyphi* A (1, 2, 12)	*S. paratyphi* B (1, 4, 5, 12)	*E. coli*	*S. sonnei*
P3B5A10	2.174	2.369	0.019	0.066	0	0.069
P3C4B2	2.391	2.614	0.003	0.011	0	0.007
P1C4C9	1.890	2.614	0	0.013	0	0
P1C3H6	2.404	2.163	0	0	0.029	0.017
P1C3A5	1.178	1.351	0.045	0.004	0.047	0.067
P3D6G5	1.986	2.340	0	0.030	0	0.007
P3B5G5	2.654	2.560	0.003	0.014	0.011	0
P3B2F7	1.914	2.557	2.281	2.105	0	0
P5C5B10	2.654	2.570	0	0.576	0.038	0.075
P5C6D1	2.654	2.227	0.030	0.820	0	0
P6C3G9	2.654	2.261	0.043	0.859	0.028	0
P5C5B2	2.654	2.519	0.035	1.899	0.073	0.065
P5C6E3	2.654	2.327	0	0.829	0.007	0.021
P1A2B10	1.327	2.184	0.789	0.674	1.064	2.612
P1A2A11	1.226	2.017	0.728	0.606	0.958	2.350

[a] The assay is described in Materials and Methods. Values expressed as A_{490} are the averages of three sets of experiments run in duplicate. The mean absorbance value for various controls was 0.07 ± 0.027.
[b] Reactivity with *S. enteritidis* was not determined.
[c] Numbers within parentheses are the O antigens expressed.

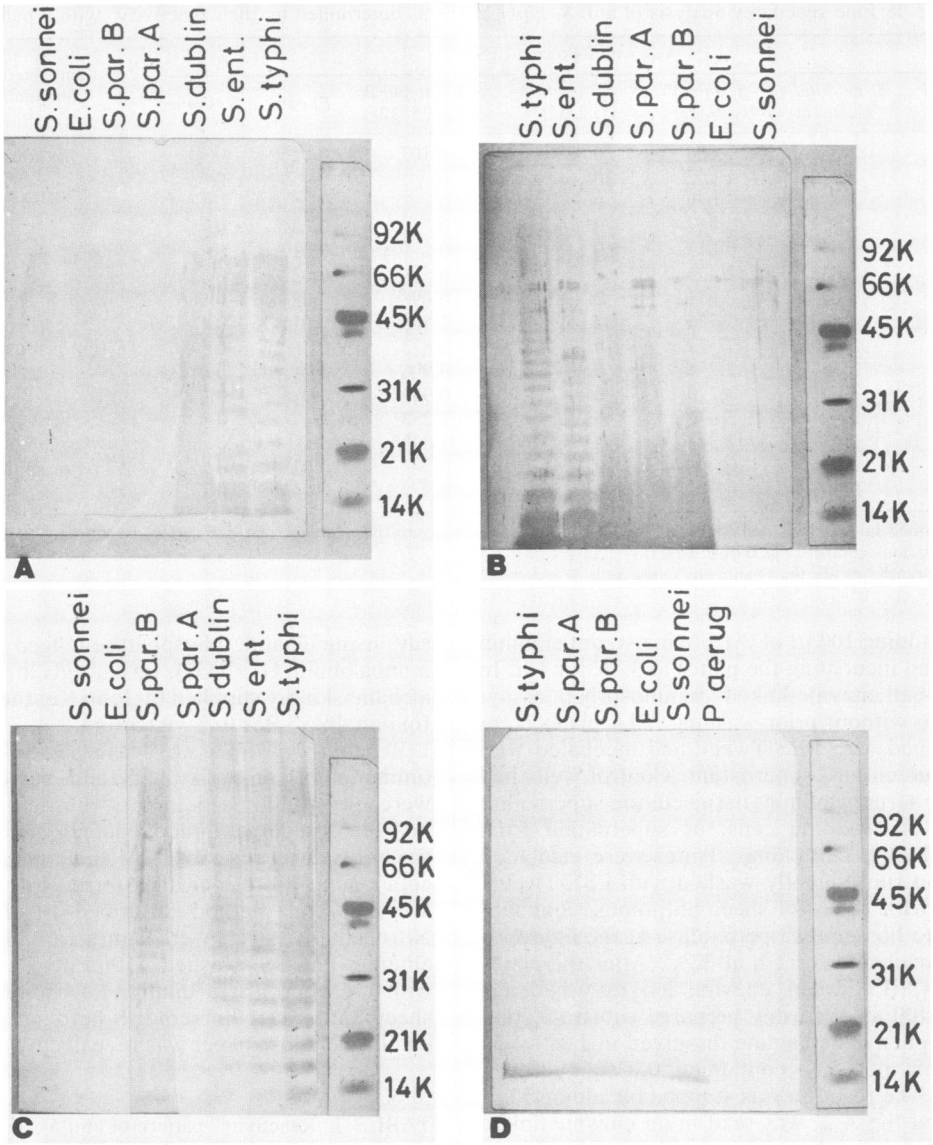

FIG. 1. Immunoblot analysis of MAbs. For panels A, B, and C, LPS were electrophoresed, blotted, and developed as described in Materials and Methods. (A) Reactivity with MAb P1C4C9; (B) reactivity with MAb P3B2F7; (C) reactivity with MAb P6C3G9; (D) electrophoresis, blotting, and development with MAb P1A2B10 of bacterial sonic extracts.

with freshly prepared 3,3-diaminobenzidine solution (0.05 mg dissolved in 1 ml of 50 mM citrate buffer, pH 5.6, containing 0.03% H_2O_2). Gels were silver stained essentially by the method of Tsai and Frasch (11).

RESULTS

Fifteen hybrid cell clones secreting MAbs were obtained from four fusions in which two different immunization regimens were used as described in Materials and Methods. Table 1 gives the reactivity of MAbs with a panel of LPS. All except two MAbs reacted with LPS isolated from *S. typhi*, *S. enteritidis*, and *S. dublin*. One MAb, P3B2F7, also reacted with LPS isolated from *S. paratyphi* A and B. Five MAbs reacted with LPS from *S. typhi*, *S. enteritidis*, *S. dublin*, and *S. paratyphi* B. These five MAbs were obtained from mice immunized with heat-killed bacteria instead of the sonic extract. None of these antibodies reacted with *S. paratyphi* A, thus differentiating two serogroups which are closely related in terms of O antigens. Two MAbs, P1A2B10, and P1A2A11, bound to antigens distinct from LPS, showing considerable cross-reactivity with *S. paratyphi* A, *S. paratyphi* B, *E. coli*, and *S. sonnei* (Table 2). All MAbs reacted with intact bacteria as well (Table 3). Four MAbs were of IgG2a(κ), five were of IgG3(κ), and six were of IgG1(κ) (Table 3).

Four MAbs, P1C4C9, P6C3G9, P3B2F7, and P1A2B10, representing four different specificity patterns, were further analyzed by Western blot. MAbs P1C4C9, P6C3G9, and P3B2F7, reactive with LPS in the direct-binding EIA, showed a typical ladder-type pattern of closely spaced bands (Fig. 1A, B, and C), corresponding well with the silver-stained pattern of LPS in the gel (Fig. 2). However, the pattern shown by P6C3G9 was slightly different from the pattern shown by P1C4C9 and P3B2F7. The reactivity in the immunoblots correlated well with the direct-binding EIA.

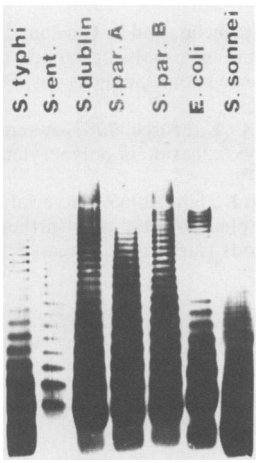

FIG. 2. LPS isolated from different bacteria were electrophoresed in a 15% sodium dodecyl sulfate–polyacrylamide gel and then silver stained.

P1A2B10 did not show any reactivity with LPS in the immunoblots as well. When reacted with bacterial sonic extracts transferred to nitrocellulose, it bound to a single fast-migrating band of around 14,000 daltons (Fig. 1D).

DISCUSSION

The results obtained from the direct-binding patterns suggest the presence of at least four different sets of MAbs. Seven MAbs reacted with LPS isolated from *S. typhi*, *S. enteritidis*, and *S. dublin*. One MAb, P3B2F7, recognizes a determinant common to *S. typhi*, *S. enteritidis*, *S. dublin*, *S. paratyphi* A, and *S. paratyphi* B. A third set of five MAbs reacts with LPS from *S. typhi*, *S. enteritidis*, *S. dublin*, and *S. paratyphi* B. *S. typhi*, *S. dublin*, and *S. enteritidis* fall in the same serogroup (serogroup D) according to the Kauffmann White scheme (5) and share O antigens 9 and 12. Figure 3 schematically represents the determinants recognized by 13 MAbs binding with LPS. Antigen 9 is represented by dideoxyhexose (tyvelose) linked to D-mannose, while antigen 12 consists of a sequence in the chain 2-D-mannose (1→4)L-rhamnose (1→3)D-galactose. *S. paratyphi* A and B share O antigen 12 with *S. typhi*. The binding patterns suggest that MAbs reacting with *S. typhi*, *S. dublin*, and *S. enteritidis* are recognizing antigen 9 and are thus specific to serogroup D, while two other sets which react with *S. paratyphi* in addition to *S. typhi* are recognizing antigen 12. The complex nature of antigen 12 has been described by Kauffmann, and it has been subdivided into 12_1, 12_2, and 12_3 (5). *S. typhi* carries all three, while *S. paratyphi* A contains 12_1 and 12_3 and *S. paratyphi* B has 12_1 and 12_2. The expression of 12_2 is variable in that it is either weakly or strongly expressed. Therefore, it seems that these two sets of MAbs, one reacting with group D salmonellae and *S. paratyphi* A and B and the other reacting with group D salmonellae and *S. paratyphi* B, are recognizing two different determinants on antigen 12. However, the possibility that P3B2F7 might be reacting to a *Salmonella* genus-specific determinant on LPS cannot be ruled out at this stage. The fourth set of MAbs, P1A2B10 and P1A2A11, shows a considerable degree of cross-reactivity, recognizing *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, *E. coli*, and *S. sonnei*. These MAbs do not react with LPS. All of the MAbs react with whole cells as well, further confirming that the MAbs are recognizing cell surface determinants.

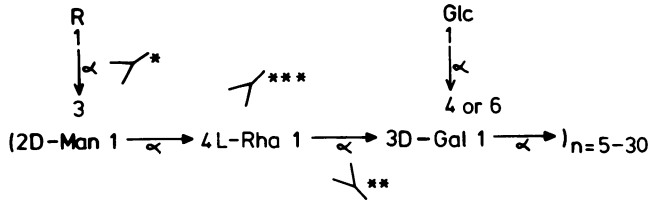

FIG. 3. Schematic representation of reactivity of MAbs with O antigens of *S. typhi*. Abbreviations: R, tyvelose; Man, mannose; Gal, galactose; Glc, glucose; Rha, rhamnose. Symbols: *, *S. typhi*-specific MAbs reactive with O antigen 9; **, MAb reacting with group D salmonellae and *S. paratyphi* A and B (O antigen 12); ***, MAbs reactive with group D salmonellae and *S. paratyphi* B (O antigen 12).

Western blot analysis showed three of the four representative MAbs, P3B2F7, P1C4C9, and P6C3G9, recognizing a ladder-type pattern of closely spaced bands with LPS. This pattern has also been demonstrated by Sidberry et al. (8) and Stoll et al. (9) with *E. coli* and *Pseudomonas* LPS and is attributed to the molecular heterogeneity of LPS molecules arising from the natural heterogeneity of sugar moieties composing the O side chains (4). There was a good correlation between the immunoblot analysis and the direct-binding EIA. MAb P1A2B10 showed a fast-migrating band common to five gram-negative bacteria. This could be either a protein molecule or a nonprotein entity which is not a part of LPS.

The data strongly indicate that the outer membrane of *S. typhi* is highly complex and presents a battery of antigenic determinants. The fact that so many MAbs generated by immunizing either the sonic extract or the whole bacilli react with O antigen reflects the immunodominant character of these antigens. Furthermore, within the O antigens separate subspecificities are discernible by virtue of the reaction with discrete MAbs.

The MAbs are currently being studied for analyzing the immune response in patients suffering from typhoid and paratyphoid fevers. This would give an insight into the fine specificity of antibodies produced during the disease.

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