Production and Characterization of Protective Human Antibodies against Shiga Toxin 1

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Hemolytic-uremic syndrome (HUS) is a serious complication which is predominantly associated in children with infection by Shiga toxin-producing *Escherichia coli* (STEC). By using HuMAb-Mouse (Medarex) animals, human monoclonal antibodies (Hu-MABs) were developed against Shiga toxin 1 (Stx1) for passive immunotherapy of HUS. Ten stable hybridomas comprised of fully human heavy- and light-chain immunoglobulin elements and secreting Stx1-specific Hu-MABs (seven immunoglobulin M(κ) [IgM(κ)] elements [one specific for the A subunit and six specific for the B subunit] and three IgG1(κ) elements specific for subunit B) were isolated. Two IgM(κ) Hu-MABs (2D9 and 15G9) and three IgG1(κ) Hu-MABs (5A4, 10F4, and 15G2), all specific for subunit B, demonstrated marked neutralization of Stx1 in vitro and significant prolongation of survival in a murine model of Stx1 toxicosis.

Hemolytic-uremic syndrome (HUS) is the leading cause of renal failure in children (11). Epidemiologically, development of HUS is associated with infection by Shiga toxin (Stx)-producing *Escherichia coli* (STEC) (14; M. A. Karmali, M. Petric, C. Lim, P. C. Fleming, and B. T. Steele, Letter, Lancet 2:1299–1300, 1983). HUS, characterized by nonimmune microangiopathic hemolytic anemia, thrombocytopenia, and acute renal dysfunction, develops in certain individuals several days following the onset of bloody diarrhea associated with food- or water-borne STEC infection (10). In the United States, the O157:H7 serotype is most frequently associated with HUS in children and the elderly (11). The risk of a child developing HUS following a bout of sporadic gastroenteritis is ~3 to 26% (18, 21, 23; W. R. Grandsen, M. A. Damm, J. D. Anderson, J. E. Carter, and H. Lior, Letter, Lancet 2:150, 1985).

Development of HUS following STEC infection is believed to be associated with the activity of two STEC-produced cytotoxins, designated Stx1 and Stx2. Although variants of Stx2 exist, Stx1 is structurally conserved and is homologous to that produced by *Shigella dysenteriae* type 1 (13). Stx1 and Stx2 are both comprised of one active (A) subunit and five binding (B) subunits. Following the binding of B subunits to globotriaosylceramide (28) and host cell uptake, the A subunits catalytically inactivate the 60S ribosomal subunits, which results in the inhibition of protein synthesis (6, 24, 25). In vivo, following systemic administration, Stx1 and Stx2 induce fatal neurological signs in piglets and mice (5, 9). Gastrointestinal infection of humans with STEC strains that produce Stx1 and Stx2 alone or in combination has been shown to induce the development of HUS (16, 19).

Presently, no effective treatment or prophylaxis for HUS is available clinically. However, passive antibody therapy holds

promise. Murine monoclonal antibodies (MAbs) against Stx have been shown to neutralize the activity of Stx1 and/or Stx2 in vitro (1, 4, 12, 20, 22, 26) and in vivo (12, 20). Using the gnotobiotic piglet model of *E. coli* O157:H7 infection, we have demonstrated that administration of either polyclonal porcine Stx2 antiserum (3) or Stx2-specific human MAbs (Hu-MABs) can prevent development of the neurological signs and lesions associated with Stx2 activity (17). Here we describe the development of a panel of Hu-MABs specific for Stx1 A and B subunits; several of these Hu-MABs neutralize Stx1-mediated activity in vitro and in vivo. Stx1-neutralizing Hu-MABs have potential clinical utility in the prevention and treatment of HUS mediated by either *S. dysenteriae* type 1 or Stx1-producing STEC. The availability of Hu-MABs against Stx1 and Stx2 provides the opportunity to administer an immunotherapeutic cocktail to individuals at risk of developing STEC-mediated HUS. Such a formulation with dual specificity would not only obviate identification of the type of Stx being produced during an STEC infection and subsequent selection of the appropriate Stx-specific treatment but would also ensure treatment coverage for those individuals infected with STEC strains producing both Stx1 and Stx2.

Stx1 and Stx1 toxoid. Stx1 was isolated, purified, and quantitated as described previously (1). Stx1 toxoid was prepared by formalin treatment of Stx1 (1).

Hybridomas and Hu-MABs. Murine hybridomas producing Stx1-specific Hu-MABs were generated by intraperitoneal (i.p.) immunization of HuMAb-Mouse mice (Medarex, San Jose, Calif.) (8) with 20 μ g of Stx1 toxoid emulsified in Freund's complete (initial immunization only) or incomplete (all subsequent immunizations) adjuvant at biweekly intervals a minimum of three times. Serum anti-Stx1 titers were determined by enzyme-linked immunosorbent assay (ELISA) on microtiter plates (Falcon catalog no. 353912; Becton-Dickinson, Bedford, Mass.) coated with 1.5 μ g of Stx1 per ml and developed with horseradish peroxidase-labeled goat anti-human immunoglobulin G(κ) [IgG(κ)]. Splenocytes from mice

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with titers of $\geq 1:800$ were fused to cells from the nonproductive murine myeloma P3X63-Ag8.653 by standard methods (7). Stable, positive clones secreting Stx1-specific IgG1(κ) Hu-MABs were identified by screening supernatants from hypoxanthine-aminopterin-thymidine-selected hybridomas by ELISA on microtiter plates coated with 1.5 μg of Stx1 per ml and developed with horseradish peroxidase-labeled goat anti-human IgM, IgG (Jackson Laboratory, Bar Harbor, Maine), or kappa chain (Bethyl Laboratories, Inc., Montgomery, Tex., or Sigma-Aldrich Co., St. Louis, Mo.). Stable, positive clones were selected by subcloning twice by limiting dilution and finally by soft-agar cloning (7).

Hu-MAB-containing ascitic fluid was prepared by injecting hybridoma cells into the peritoneal cavity of pristane (Sigma-Aldrich Co.)-primed ICR-SCID mice (Taconic, Germantown, N.Y.). Hu-MAB concentrations in ascitic fluid were determined relative to isotype-matched concentration standards (Sigma, St. Louis, Mo.) by ELISA (27).

To identify potential murine-human hybrid Hu-MABs (8) and determine the species-specific heavy- and light-chain isotypes of all Hu-MABs isolated, two ELISAs were performed. In one ELISA, each Hu-MAB was added to 15 wells of microtiter plates (Costar, Corning, N.Y.) coated with 1 μg of Stx1 per ml. Each well was then developed with alkaline phosphatase (AP)-labeled goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA (kappa or lambda chain) or AP-labeled anti-human IgM, IgG1, IgG2, IgG3 or IgG4 (kappa or lambda chain) (Southern Biotechnology Associates, Inc., Birmingham, Ala.). In the second ELISA, each Hu-MAB was added to eight wells of microtiter plates (Costar) coated with goat anti-human kappa chain (Southern Biotechnology Associates). Each well was then developed with AP-labeled anti-human IgM, IgG1, IgG2, IgG3, IgG4, or IgA (kappa or lambda chain; Southern Biotechnology Associates).

The Stx binding specificity of each Hu-MAB was determined with a sandwich ELISA in which microtiter plates coated with the murine Stx1- or Stx2-specific MABs 4D3 or 3D1 (1) at 5 $\mu\text{g}/\text{ml}$ were used to capture Stx1 or Stx2 (1 $\mu\text{g}/\text{ml}$), respectively. Hybridoma culture supernatants containing individual Hu-MABs were plated in duplicate on pairs of plates containing Stx1 or Stx2. The plates were then developed with AP-labeled goat anti-human kappa chain (Southern Biotechnology Associates).

The Stx1 subunit specificity of each Hu-MAB was determined by Western blot analysis. Stx1, comprised of one A subunit of ~ 32 kDa and five B subunits of ~ 7.7 kDa each (13), can be cross-linked with dimethylpimelimidate (Pierce Chemical Company, Rockford, Ill.) to create a mixture containing B subunit multimers and the A subunit bound to one to five B subunits. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% acrylamide slab gel and electrophoretic transfer to a nylon membrane (Immobilon-P; Millipore, Bedford, Mass.), binding patterns of individual Hu-MABs to the various A-B complexes and A and B subunit monomers or multimers can be examined. MABs with specificity for the B subunit bind the B subunit monomers or multimers and the A-B complexes; MABs with specificity for the A subunit bind the A-B complexes but do not bind B subunit monomers or multimers (2, 17).

In vitro and in vivo Stx1 neutralization. The ability of each Stx1-specific Hu-MAB to neutralize the effects of purified Stx1 was examined both in vitro with a HeLa cell cytotoxicity assay and in vivo with a murine Stx1 neutralization assay. For the in vitro assay, HeLa cells were plated at $2 \times 10^5/\text{ml}$ in McCoy's 5A medium (Mediatech, Inc., Herndon, Va.) plus 10% fetal calf serum (Harlan Bioproducts for Science, Inc., Madison, Wis.) and incubated overnight at 37°C in 5% CO_2 . Each Hu-MAB was serially diluted 1:2 at concentrations from 12.5 to 0.0061 $\mu\text{g}/\text{ml}$, and each dilution was incubated for 30 min at room temperature with 10 ng of Stx1 per ml. Following removal of the medium, Stx1-Hu-MAB mixtures were transferred to HeLa cell monolayers and incubated overnight at 37°C in 5% CO_2 . The proportion of surviving cells following exposure to Stx1 with or without each Hu-MAB was determined spectrophotometrically following crystal violet staining (15). Each assay was performed independently a minimum of three times; the results at a selected data point were averaged.

For the in vivo assay, 3- to 4-week-old female Swiss Webster mice were divided into groups of 6 to 10 mice. Fifty micrograms of Stx1-specific Hu-MAB in 1 ml of phosphate-buffered saline (PBS) or 1 ml of PBS alone (control) was administered i.p. to each of 6 to 10 3- to 4-week-old Swiss Webster mice. Approximately 18 h later, 0.5 μg of Stx1 (the minimum dose required to induce 100% mortality; data not shown) was administered intravenously via the lateral tail vein. The mice were observed twice daily for survival. Experiments were terminated 12 days following Stx1 challenge. Survival data were analyzed by both parametric (log rank test) and nonparametric (Wilcoxon test) methods by using the software program Statistica (version 4.1; Statsoft, Inc., Tulsa, Okla.). Both methods yielded comparable *P* values. Comparisons were considered significant when *P* was < 0.05 .

Eleven stable hybridomas secreting Stx1-specific Hu-MABs were isolated. Ten hybridomas [seven of the IgM(κ) and three of the IgG1(κ) isotypes] secreted Stx1-specific Hu-MABs fully comprised of human heavy- and light-chain immunoglobulin elements (Table 1). Although the fusion was screened with human heavy- and light-chain-specific reagents, one hybridoma (1E2) was isolated that secreted an Stx1-specific hybrid Hu-MAB comprised of a murine IgG2a heavy chain and a human kappa light chain. Such hybrids can arise, because although the endogenous murine immunoglobulin loci are inactivated, they are still present in the HuMAB-Mouse animal (8). Only the 10 Hu-MABs comprised of fully human immunoglobulin elements were studied further. Of the IgM(κ) Hu-MABs, one (7E12) bound the Stx1 A subunit and six (2D9, 15G9, 1B10, 8A5, 14C9, and 14H3) bound the Stx1 B subunit. Of the three IgG1(κ) Hu-MABs (5A4, 10F4, and 15G2), all bound the Stx1 B subunit (Table 1). As determined by ELISA, cross-reactivity with Stx2 was not observed. This is in contrast to results for previously described panels of murine Stx-specific MABs in which cross-reactivity with Stx1 and Stx2 has been observed via either Western blotting (20) or ELISA (1). However, as with previously described panels of Stx1-specific murine MABs, both A and B subunit-specific IgM(κ) and IgG1(κ) Hu-MABs were isolated (1, 12, 20).

In an effort to determine which Hu-MABs may have potential therapeutic utility, we evaluated the ability of each Hu-MAB to neutralize purified Stx1 both in vitro and in vivo.

TABLE 1. Summary of Stx1 Hu-MAb isotypes, subunit specificities, and rates of in vivo and in vitro neutralization of Stx1^a

Hu-MAb	Isotype	Stx1 subunit specificity	% neutralization of Stx1 (avg $\pm$ SD) ^b	Murine survival ^c	
				Days (avg $\pm$ SD)	<i>P</i> value
Group 1					
2D9	IgM(κ)	B	100.0 $\pm$ 0	12.0 $\pm$ 0 ^d	0.00005
5A4	IgG1(κ)	B	88.7 $\pm$ 1.15	12.0 $\pm$ 0 ^e	0.00005
10F4	IgG1(κ)	B	99.7 $\pm$ 0.14	12.0 $\pm$ 0 ^e	0.00005
15G2	IgG1(κ)	B	96.2 $\pm$ 1.59	12.0 $\pm$ 0 ^d	0.00005
15G9	IgM(κ)	B	96.9 $\pm$ 5.03	12.0 $\pm$ 0 ^e	0.00005
Group 2					
1B10	IgM(κ)	B	63.3 $\pm$ 18.46	9.55 $\pm$ 3.95 ^d	0.00071
7E12	IgM(κ)	A	75.7 $\pm$ 9.29	9.08 $\pm$ 4.52 ^d	0.03389
8A5	IgM(κ)	B	57.7 $\pm$ 10.26	5.39 $\pm$ 3.77 ^d	0.13821
14C9	IgM(κ)	B	76.3 $\pm$ 13.35	6.80 $\pm$ 4.49 ^d	0.14342
14H3	IgM(κ)	B	77.3 $\pm$ 5.91	7.95 $\pm$ 4.28 ^d	0.00268

^a Stx1 Hu-MABs have been sorted into two groups based on their average percentages of neutralization of Stx1 in vitro and their abilities to prolong average survival in vivo. Group 1 includes those Stx1 Hu-MABs with in vitro neutralization values of $>85\%$ and average survival prolongations of >10 days. Group 2 includes those Stx1 Hu-MABs with in vitro neutralization values of $<84\%$ and average survival prolongations of <10 days.

^b Neutralization of 1 ng of Stx1 in vitro in the presence of 39.1 ng of Hu-MAB was determined by the Hela cell cytotoxicity assay. The TC₅₀ is 1.05×10^8 per mg of Stx1.

^c Experiments were terminated on day 12 (number of mice, 6 to 10). *P* values were calculated for the comparison of the average survivals of PBS-treated control groups and Hu-MAB-treated groups by parametric (log rank) and nonparametric (Wilcoxon) analyses. Comparable *P* values were obtained with both analyses. The table shows *P* values obtained by Wilcoxon analysis.

^d Average survival of PBS control ($\pm$ standard deviation), 3.45 ± 0.64 days.

^e Average survival of PBS control ($\pm$ standard deviation), 3.55 ± 0.93 days.

Based on their relative percentages of in vitro neutralization with specific quantities of Hu-MABs and Stx1 (39.1 and 1 ng, respectively) and the results of the in vivo Stx1 neutralization assays, the Stx1-specific Hu-MABs were divided into two categories, namely those which were highly neutralizing ($>85\%$ neutralization in vitro and prolongation of average survival to >10 days) and those which were moderately to poorly neutralizing ($<85\%$ neutralization in vitro and prolongation of average survival to <10 days). Hu-MABs 2D9, 5A4, 10F4, 15G2, and 15G9 were found to be highly neutralizing (Table 1, group 1), whereas Hu-MABs 1B10, 7E12, 8A5, 14C9, and 14H3 were found to be only moderately to poorly neutralizing (Table 1, group 2). Consistent with previous findings by Padhye et al. (20) and Islam et al. (12), who used murine MABs with specificity for Stx1, the ability to neutralize Stx1 in vitro correlated with the ability to neutralize Stx1 in vivo. This is in contrast to our recent findings for Stx2-specific Hu-MABs in which we demonstrated that in vitro neutralization of Stx2 did not necessarily ensure the ability to neutralize Stx2 in vivo (17). Of the five Hu-MABs which neutralized $>85\%$ of the Stx1 in vitro and prolonged murine survival by >10 days, two were of the IgM(κ) isotype and three were of the IgG1(κ) isotype, indicating that Hu-MABs of either isotype can neutralize the effects of Stx1. Although only one Stx1 A subunit-specific Hu-MAB (7E12) was isolated, the Hu-MABs most effective at neutralizing Stx1 both in vitro and in vivo are specific for the B subunit.

Stx1-specific murine MABs have previously been shown to neutralize the effects of Stx1 in vivo and/or in vitro (12, 20, 26); however, species incompatibility precludes the use of these MABs in humans. The availability of species-compatible, Stx1-neutralizing Hu-MABs now facilitates the opportunity to passively treat or prevent Stx1-mediated HUS development associated with either *S. dysenteriae* type 1 or STEC infection in susceptible individuals. These Stx1-specific Hu-MABs, furthermore, could be combined with previously described protective

Stx2-specific Hu-MABs (17) to create an immunotherapeutic cocktail with dual specificity for Stx1 and Stx2. Such a formulation could be administered to prevent HUS development in susceptible STEC-infected patients and contacts without the need to toxinotype the STEC strain and select the appropriate Stx-specific treatment. This would also ensure treatment coverage for all STEC-infected individuals and their contacts regardless of whether the strain produced Stx1, Stx2, or both.

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