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Comparison of Pertussis Toxin (PT)-Neutralizing Activities and Mouse-Protective Activities of Anti-PT Mouse Monoclonal Antibodies

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An assay of neutralizing activity against CHO cell-clustering activity, one of the most common assays for anti-pertussis toxin antibodies, did not correlate well with an assay of the protective activities of monoclonal antibodies. A better correlation between neutralization of leukocytosis-promoting or islet-activating activity and mouse protection against aerosol challenge was seen.

Pertussis toxin (PT) is the main virulence factor produced by *Bordetella pertussis* and, as a protective antigen, is the most important component of an acellular pertussis vaccine developed in Japan (14, 17). PT is a very complicated hexamer consisting of five different subunits designated S1 through S5 and has diverse biological and toxic activities such as ADP-ribosylating (ADPR), hemagglutinating, T-cell-mitogenic, CHO cell-clustering (CC), histamine-sensitizing, islet-activating (IA), and leukocytosis-promoting (LP) activities in vitro and in vivo. The toxicity of PT is believed to be generated by the active (A) promoter, S1, which is an ADPR enzyme, in cooperation with the binding (B) oligomer, which contains S2, S3, S4, and S5 in a molar ratio of 1:1:2:1. S2 and S3 have an amino acid sequence homology of about 70% (5, 6). Although S1 is not required for hemagglutinating or T-cell-mitogenic activity since the S24 and S34 dimers show hemagglutinating activity and the B oligomer shows T-cell-mitogenic activity (7, 9), a whole molecule of PT with evident ADPR activity of S1 is regarded as indispensable for most of the other activities because no combination lacking any subunit or PT whole molecule lacking enzyme activity because of a mutation of S1 has generated any of these toxic activities, such as CC, IA, and LP activities (8, 9, 11, 16, 18). If immunoprotectivity of the pertussis vaccine depends mainly on PT neutralization with antibodies against PT, then protectivity should be correlated with the PT-neutralizing activity of the antibody.

We have developed mouse monoclonal antibodies (MAbs) against various parts of the PT molecule and have been using these MAbs as tools for analysis of the toxicity, immunoprotectivity, and structure of PT in vitro and in vivo (9, 12, 13). In this study, the abilities of 32 different anti-PT mouse MAbs to protect mice against aerosol or intracerebral (i.c.) challenge with virulent *B. pertussis* organisms and neutralize ADPR, CC, LP, and IA activities of PT were compared in order to determine which PT activity among the various biological activities should be neutralized with the MAbs to protect mice and to find better laboratory tests to predict the protectivity of the antibodies. Of the 32 MAbs, 20 have been investigated with regard to mouse protection (9, 12). However, results of PT neutralization studies have been reported for only 5 of the 20 MAbs, which were roughly correlated with mouse protectivity (9, 13). To determine the more general characteristics of the relationship between the neutralization and protection abilities of anti-PT antibody, 12 new anti-B-oligomer MAbs, 1 anti-S23 MAb (3F6), 2 anti-S2 MAbs (3A12 and 2H3), 4 anti-S3 MAbs (4G5, 2E12, 6F8, and 10B8), 4 anti-S4 MAbs (7F2, 6B3, 9C6, and 6D6), and 1 anti-S5 MAb (7E3) were generated and purified as reported previously (12) and investigated in this study together with the 20 MAbs generated previously. The specificities of binding of the MAbs to each subunit were determined by immunoblotting analysis and enzyme-linked immunosorbent assay (ELISA) using PT and its subunits as reported previously (12, 13, 16, 19). As a reference antibody, anti-PT mouse polyclonal antibody M6 (5,500 PT ELISA units per ml) was used to determine the binding activities of the MAbs to PT and to calculate the number of PT ELISA units of the MAbs by the parallel line assay method as described in a previous article (12). The glycerinated purified PT (200 μg/ml) was kept at −20°C without freezing and used as the coating antigen for the PT ELISA, the reference toxin for the assay of PT activity, and the test toxin for the assay of the toxin-neutralizing activities of the antibodies.

Mouse protection tests were carried out as described previously (10, 12, 15). In the aerosol challenge system, 6-day-old suckling mice were immunized passively with 20 μg of the purified antibody by intraperitoneal injection 2 h before aerosol challenge with *B. pertussis* 18323. Leukocyte concentrations and body weight were measured every week for 5 weeks in order to confirm the illness and deaths caused by pertussis infection. In the i.c. challenge system, 6-week-old mice immunized intraperitoneally with 40 μg of the antibody were challenged with *B. pertussis* 18323 (50,000 cells per mouse in a 25-μl challenge suspension volume) 3 h after antibody administration and then observed for 2 weeks. Although the mouse protectivities of 20 MAbs were determined and reported previously (12), these MAbs were assayed again together with the new 12 MAbs, and the survival rate was recalculated.

Binding and neutralizing activities of MAbs to PT were assayed by the methods described in previous articles (12, 13), and mean values of repeated experiments are given in this article. For assay of anti-ADPR activity in a cell-free system, 2 μg of PT and 20 μg of the purified MAb were preincubated, and the residual ADPR activity of the mixture was assayed by determining the incorporation of [^{32}P]ADP-

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ribose into the CHO cell membrane protein. The neutralization activity was expressed as the percent inhibition of the incorporation of ^{32}P. The incorporation of ^{32}P into a 41-kDa protein in the membrane was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography as described in a previous report (13). The effect of MAbs on the ADPR activity of PT in intact CHO cells was investigated as follows. An overnight culture of CHO cells in a 24-well plate (10^5 cells per well) was washed with phosphate-buffered saline PBS (0.15 M NaCl plus 10 mM K-Na phosphate buffer, pH 7.3) and treated in culture medium (F12) containing 1% fetal calf serum at 37°C for 3 h with a mixture of PT (2 μg) and antibody immunoglobulin G (2, 20, or 200 μg as indicated in Fig. 1) which had been preincubated for 30 min. As a control, CHO cells were incubated with the culture medium alone or PT alone. The cells were washed again with PBS and were scraped off and lysed by adding 50 μl of a reaction buffer which consisted of 25 mM Tris hydrochloride (pH 7.5), 56 mM thymidine, 5.6 mM ATP, 0.56 mM GTP, and 5.6 mM EDTA. The cell lysate (40 μl) was incubated with 20 μl of PT (2 μg) again in the presence of [^{32}P]NAD and 10 mM dithiothreitol at a final volume of 75 μl at 37°C for 30 min to test for ADP-ribosylation with [^{32}P]NAD of the membrane protein, which had not been ribosylated with cold NAD in vivo. The reaction mixture was centrifuged at 12,000 × *g* for 5 min, and the pellet was washed twice with 25 mM Tris hydrochloride (pH 7.5) containing 2.5 mM $MgCl_2$ and then dissolved in 10 μl of 10% SDS–0.1 M Tris-HCl (pH 8.0) containing 8 M urea and 100 mM dithiothreitol. Electrophoresis on a 10% polyacrylamide slab gel was followed by exposure of Fuji RX film to the dried gel for 18 h. Anti-CC activity was assayed at least four times and expressed as the minimum amount of the MAb which yielded a negative reaction against the clustering of CHO cells with 15 pg of PT. Anti-LP activity was assayed by counting peripheral leukocytes of five mice 3 days after intravenous injection of a mixture of 0.1 μg of PT and 10 μg of the purified MAb. The net leukocyte count was obtained by subtraction of the leukocyte count of normal mice, and the geometric mean was calculated by using the net leukocyte counts from five mice, as described previously (13). The neutralization activity was expressed as percent inhibition of leukocyte increase by the MAb with 0.1 μg of PT. The mean value of the percent inhibition from at least three experiments was calculated. For anti-IA activity, the mice were starved overnight after the leukocyte count for the anti-LP assay. Blood samples taken from the five mice 15 min after intraperitoneal injection of 50% glucose were pooled, and insulin in the sera was measured by ELISA as described previously (13). The neutralization activity was expressed as the percent inhibition of insulin increase with 0.1 μg of PT by the MAb.

Table 1 shows the PT-binding, PT-neutralizing, and mouse-protective activities of the 32 purified MAb immunoglobulin G preparations. The MAbs are listed in order of protectivity against the aerosol challenge in each group of antibodies recognizing the same subunit. Most anti-S1 and anti-S4 MAbs which showed high protectivity in the aerosol system also protected mice significantly against the i.c. challenge. However, antibodies against S2 and/or S3 subunits showed little or no protection in the i.c. challenge system, even though they showed high protectivity against the aerosol challenge. Anti-S5 MAb 7E3 did not show any anti-PT activity except binding to PT.

All anti-S1 MAbs inhibited ADP-ribosylation of the CHO cell membrane protein with PT regardless of their other PT-neutralizing or mouse-protective activities; E2E, 3F10, 11D9, and 4D10, which neither neutralize CC, LP, or IA activity nor protect mice, had high anti-ADPR activity levels. The effect on ADP-ribosylation with PT in intact CHO cells was also investigated with protective MAb 1B7 and nonprotective MAb 3F10. As shown in Fig. 1, the decrease in ADP-ribosylation of the cellular protein by prior treatment of the cells with PT with or without the MAbs was assayed by subsequent incubation with PT and [^{32}P]NAD. MAb 3F10 did not inhibit ADP-ribosylation with PT in the intact cell culture system, as shown by the absence of radioactive ADP-ribose on the protein which had already been ADP-ribosylated with cold NAD by initial treatment with a PT-3F10 mixture as well as with PT alone in vivo. On the other hand, since 1B7 prevented ribosylation of the protein with PT in vivo, the protein was ribosylated with radioactive ADP-ribose by the second treatment with PT in vitro. This result showed that the anti-ADPR activity presented in Table 1 does not necessarily reflect the anti-ADPR activities of the MAbs in vivo. Therefore, it is impossible to predict the anti-toxic or protective activities of antibodies by their anti-ADPR activities in a cell-free system.

The CC activity of PT was neutralized less effectively with anti-S1 antibodies than with antibodies against S2 and/or S3 subunits. Among the anti-S1 MAbs, only 1B7, 1D7, and 3F11 neutralized the CC activity. 10D6 and 8G4 did not neutralize the CC activity despite their high anti-LP and anti-IA activities and high mouse protectivity. On the other hand, almost all MAbs against S2 and/or S3 subunits showed rather high levels of anti-CC activity, even if they did not show significant protectivity. Among the anti-S4 MAbs, all protective MAbs neutralized CC activity. The assay of anti-CC activity is considered to be the most convenient and common method for determining the PT-neutralizing activity of the antibody. As shown in Table 1, however, anti-CC activity seems to depend mainly on anti-B-oligomer antibodies and not on anti-S1 antibodies in the sera and is not necessarily correlated with mouse protectivity. For evaluation of pertussis vaccine efficacy, PT-binding and PT-neutralizing activities of the sera of vaccinees are generally determined by ELISA and CC assay, respectively, but it is possible that these antibody activities are not correlated with vaccine efficacy. Therefore, prediction of protectivity by anti-CC activity of antibodies is difficult.

The LP and IA activities of PT were inhibited by most of the protective MAbs. Although there were some exceptions, a better correlation was seen between neutralization of LP or IA activity and mouse protection in the aerosol challenge system than in the i.c. challenge system. Since dose-response regression lines of the percent neutralization of LP and IA activities were not parallel for all MAbs, as explained previously (9, 13), it was impossible to calculate the relative neutralizing activity of each MAb by using a reference antibody such as M6 by the parallel line or reference line assay method. Although anti-LP and anti-IA activities expressed as the inhibition percentages for the activities of 0.1 μg of PT with 10 μg of the MAb in Table 1 were almost the same as the survival rates of mice in the aerosol infection experiment, some MAbs maintained the same inhibition percentage for LP and IA while others showed sharp drops in the neutralization rates when 1 μg of the MAb was used instead (data not shown). Therefore, the value of the neutralizing activity of the MAbs varies, depending on the calculation method and the method of expression of the results. The difference between anti-CC and anti-LP or anti-IA activities of the MAbs shown in Table 1, however,

TABLE 1. Relationship between PT neutralization and mouse protection with MAbs

Antibody	Binding (PT ELISA units/mg)	Neutralization for:				Mouse protection [% survival (S/T)[a] with]:	
		ADPR[b] (%)	CC[c] (ng)	LP[d] (%)	IA[d] (%)	Aerosol[e]	i.c.[f]
Anti-S1 MAb							
1B7	7,400	80	93	97	85	100 (25/25)	83 (25/30)
1D7	4,500	31	1,390	70	53	96 (23/24)	40 (12/30)
3F11	740	84	4,170	55	45	88 (22/25)	37 (11/30)
10D6	14,000	62	>12,500	82	58	80 (20/25)	30 (9/30)
8G4	8,500	94	>12,500	79	70	68 (17/25)	7 (2/30)
E1E	5,500	80	12,500	45	45	55 (12/22)	20 (6/30)
E2E	1,000	77	12,500	20	20	36 (9/25)	0 (0/30)
3F10	4,400	60	>12,500	20	20	29 (7/24)	0 (0/20)
11D9	700	95	>12,500	<10	<10	10 (2/20)	0 (0/10)
4D10	1,780	92	>12,500	<10	<10	10 (2/20)	0 (0/20)
Anti-S23 MAb							
11E6	2,850	29	21	90	82	92 (23/25)	7 (2/30)
10C9	1,240	37	185	77	69	88 (22/25)	15 (3/20)
10B5	1,500	27	185	67	67	84 (21/25)	0 (0/30)
G9A	1,900	0	185	39	42	57 (13/23)	0 (0/30)
3F6	8,000	ND[g]	12	66	80	31 (5/16)	10 (1/10)
Anti-S2 MAb							
3A12	4,000	ND	248	83	67	84 (16/19)	20 (2/10)
2H3	800	ND	1,471	52	43	77 (13/17)	10 (1/10)
9G8	10,000	2	185	37	35	68 (17/25)	20 (4/20)
Anti-S3 MAb							
7E10	9,350	0	62	77	69	54 (15/28)	5 (1/20)
7G11	1,260	6	321	48	52	52 (14/27)	10 (3/30)
4G5	5,000	ND	231	65	70	35 (6/17)	0 (0/10)
2E12	3,500	ND	1,087	28	28	31 (5/16)	0 (0/10)
6F8	3,000	ND	893	15	41	27 (4/15)	0 (0/10)
10B8	6,000	ND	4,167	17	25	18 (3/17)	0 (0/10)
Anti-S4 MAb							
7F2	3,200	ND	63	72	95	79 (11/14)	80 (8/10)
6B3	2,700	ND	236	44	76	64 (9/14)	50 (5/10)
6G8	1,300	27	4,167	43	52	43 (12/28)	0 (0/20)
9F3	1,600	0	>12,500	20	18	10 (3/30)	0 (0/20)
1H2	540	10	>12,500	<10	<10	10 (3/30)	0 (0/20)
9C6	370	ND	>12,500	<10	<10	10 (2/20)	0 (0/20)
6D6	1,100	ND	>12,500	<10	<10	5 (1/20)	0 (0/20)
Anti-S5 MAb 7E3	170	ND	>12,500	<10	<10	10 (1/10)	0 (0/10)
PAb[h] M6	1,100	100	62	56	54	58 (21/36)	27 (8/30)
Normal mouse IgG[i]	0	0	>12,500	<10	<10	8 (3/36)	0 (0/20)

[a] Survival rates are indicated by percentages and in parentheses by the ratio of the number of mice surviving (S) to the total number of mice (T).
[b] Percent inhibition of ^{32}P incorporation into the cell membrane protein with PT.
[c] Minimum amount of antibody which neutralized the CC activity of PT.
[d] Percent inhibition of increase in leukocytes (for LP) or insulin (for IA) in mice treated with PT.
[e] Survival rates with 20 μg of MAb per mouse 5 weeks after challenge.
[f] Survival rates with 40 μg of MAb per mouse 2 weeks after challenge.
[g] ND, not determined.
[h] PAb, polyclonal antibody.
[i] IgG, immunoglobulin G.

should be explained according to the different mechanisms of CC and LP or IA activities of PT and not by differences in their calculation or expression methods. Therefore, caution is required for evaluation of these PT-neutralizing activities. Although several research groups have described characteristics of anti-PT MAbs (1–4), it is difficult to compare the characteristics and activities of their MAbs with those of ours because of different assay methods and different criteria for the activities. For example, it may be possible to say that the strong CC-neutralizing anti-S1 MAb 3CX4, classified as A (3), does not have a higher mouse-protective activity level than the weak CC-neutralizing MAb 6FX1, classified as B, which has the same level of LP-neutralizing activity as 3CX4 (4).

The results described above show that although there was a better correlation between the abilities of the MAbs to neutralize LP or IA activity of PT and to protect mice than between their abilities to neutralize CC activity and to

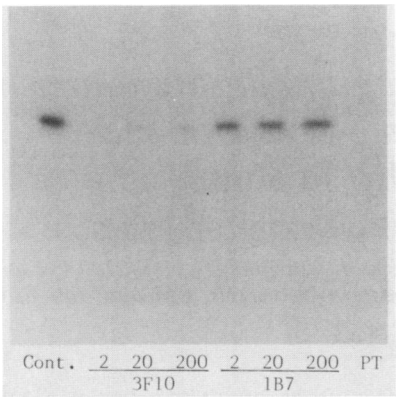

FIG. 1. Effect of MAbs on ADPR activity of PT in intact CHO cells. Intact CHO cells were treated with the medium alone as a control (Cont.); mixtures of 2 μg of PT and 2, 20, or 200 μg of the MAbs as indicated; or PT alone (PT). After treatment, the cell lysates were incubated with PT and [^{32}P]NAD and applied to an SDS-polyacrylamide gel. ^{32}P incorporation into the 41-kDa protein was monitored by autoradiography.

protect mice, estimation of the protective properties of MAbs based on a single biological system such as anti-LP activity may not provide an accurate estimate of the protectivity of the MAbs. In this study, individual MAbs were used to compare mouse protectivity and PT neutralization. Further analysis using not only individual MAbs but also combinations of MAbs will be necessary to apply these MAbs as models or reference antibodies for polyclonal immune serum to predict its protectivity. Finally, it is important to clarify the level of correlation between mouse-protective or PT-neutralizing activity and human-protective activity of anti-PT antibody.

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