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Mutant of *Bordetella pertussis* Which Lacks Ability to Produce Filamentous Hemagglutinin

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Mutant cultures that lacked the ability to produce filamentous hemagglutinin were obtained by UV irradiation of *Bordetella pertussis* strain 18-323. Various biological activities of these mutants were compared with those of the parent culture. It was found that the leukocytosis-promoting, histamine-sensitizing, and mouse-protecting activities of the mutants were similar to those of the parent strain but that the virulence to mice was lower. The results seemed to rule out fimbrial hemagglutinin as a protective antigen when the ability to protect mice was measured by the intracerebral challenge test.

Keogh and North and Keogh et al. (9, 10) reported that suspensions of *Bordetella pertussis* cells were capable of agglutinating erythrocytes from various animal species. They also found that the supernatants of liquid cultures from *B. pertussis* contained hemagglutinin (HA), and they considered this substance to be responsible for inducing active immunity against *B. pertussis* infection. Pillemer (17), Thiele (22), and Masry (12) reported that the mouse protective antigen and HA of *B. pertussis* are not identical. Arai and Sato (1) and Sato et al. (19) found that *B. pertussis* contains two HAs. One HA, called fimbrial HA (FHA), which had filamentous structure (2 by 40 nm), was nontoxic and protected mice from infection with *B. pertussis*. The other, called lymphocytosis-promoting factor HA, had a spherical structure (6 nm in diameter), was toxic and induced histamine sensitivity but did not protect mice from infection at the doses tested.

Munoz and Bergman (14) demonstrated that pertussigen (histamine-sensitizing factor, leukocytosis-promoting factor) protects mice from intracerebral infection with *B. pertussis* but, contrary to the findings of Sato et al. (19), FHA does not.

In this work we isolated mutants from *B. pertussis* that did not produce FHA but had other biological activities found in the parent strain. The results of this investigation are presented in this paper.

MATERIALS AND METHODS

Bacterial strains. The following strains of *B. pertussis* obtained from our lyophilized stock cultures were employed. (i) Strain 18-323, agglutinogen type 1.2.4, was a subculture of the original mouse virulent culture described by Kendrick et al. (8). This culture was originally described to contain agglutinogens 1.2.3.4.5.6, but our strain contained only agglutinogen 1.2.4. The other strains were: (ii) the agglutinogen type 1 NKW (Nakano) strain (24); (iii) the agglutinogen type 1.2.3.4 Maeno strain (5, 16); and (iv) the Sakairi strain, which lacks K agglutinogens and is a phase III strain according to Kasuga's terminology (5–7). This phase has been called phase IV or rough by various workers (11, 20, 23). Kasuga et al. (7), however, showed clearly that their phase III strains differed from rough or phase IV strains in having a serologically distinct O antigen in common with phase I and intermediate strains. The rough strains have serologically different O antigens (5).

These cultures were plated on Bordet-Gengou (BG) agar (2), and their phase and agglutinogen types were checked with cell suspension made in phosphate-buffered saline. The presence of K agglutinogens was checked with anti-Maeno strain antiserum absorbed with autoclaved Maeno strain cells to remove O agglutinins. The agglutinogen types were determined by agglutination reaction with specific antisera prepared as described by Eldering et al. (3).

To obtain cells for irradiation studies, the 18-323 strain was grown in a modified Cohen-Wheeler medium (4) incubated at 36°C for 24 h under constant shaking (Taiyo Co., Japan; Monoshin type II shaker). Cells from these cultures were spread on the surface of BG agar plates and then irradiated with UV light (Toshiba lamp GL 150) for different lengths of time. After irradiation the plates were incubated at 36°C, and the colonies that developed were subcultured individually on fresh BG plates for further studies.

Antisera. Anti-K agglutinogen sera were produced in rabbits immunized with *B. pertussis* strain 18-323 cells, as described by Kasuga et al. (5). When the agglutinin titers reached adequate titers (over 1/2000), the rabbits were bled, and the sera were collected and then absorbed with autoclaved 18-323 strain cells. Anti-O sera were prepared by immunizing rabbits with cells of the Sakairi phase III strain of *B. pertussis*, as described by Kasuga et al. (5). Typing antisera for

agglutinogen factor 1, 2, 3, and 4 were prepared as described by Eldering et al. (3).

Mice. Four- to five-week-old female mice of the ddY strain were used to assay for virulence, leukocytosis-promoting, histamine-sensitizing, and mouse-protecting activities.

Biological assays. The cell suspensions used for the various biological assays described below were made by culturing *B. pertussis* on BG plates incubated for 48 h at 36°C. The cells were collected and suspended to contain 10×10^9 cells per ml in the proper diluent and further diluted as required.

(i). HA activity. HA activity was determined by the method of Masry (12) on cell suspensions made in saline.

(ii). Agglutinin activity. Agglutinin activity was tested on dilutions of sera (0.25 ml) to which an equal volume (0.25 ml) of cells suspended in phosphate-buffered saline containing 0.3% Formalin was added. After mixing, the suspensions were incubated 2 h at 36°C and overnight at 4°C before the results were recorded.

(iii). Dermonecrotic activity. Dermonecrotic activity was measured on suspensions of cells made in phosphate-buffered saline to contain 10×10^9, 10×10^8, and 10×10^7 cells per ml. A 0.1-ml amount of each suspension was given intradermally on the shaven backs of guinea pigs. The dermonecrotic reactions were recorded at 24 and 48 h after the administration of the cell suspension.

(iv). Virulence. Virulence was determined with cells suspended in 1% Casamino Acids solution. From this suspension 10-fold dilutions were made in 1% Casamino Acids solution, and 0.025 ml of each suspension was given intracerebrally to groups of 10 mice. The animals were observed for 2 weeks, and the deaths and survivals were recorded. The 50% lethal doses (LD_{50}'s) were calculated by the method of Reed and Muench (18).

(v). Leukocytosis-promoting activity. Leukocytosis-promoting activity was measured by making a suspension of cells (5×10^9/ml) in phosphate-buffered saline. This suspension was heated at 56°C for 30 min, and then 0.2 ml was given intravenously into each of five mice. Three days later the total leukocyte count was made with the Coulter Counter on blood obtained by clipping the end of the tail.

(vi). Histamine-sensitizing activity. Histamine-sensitizing activity was assayed in cell suspensions made as for the assay of leukocytosis-promoting activity. The mice received intravenously 0.2 ml of the cell suspension and were challenged intraperitoneally 3 days later with 3 mg of histamine dihydrochloride dissolved in saline. Deaths were recorded 1 h later.

(vii). Protective activity. Protective activity was measured on cell suspensions made in phosphate-buffered saline containing 0.01% thimerosal. Mice weighing 14 to 18 g were used. Three fivefold dilutions of the cell suspensions were tested (5×10^9, 1×10^9, and 2×10^8 cells per ml). Each mouse in a group of 16 mice received intraperitoneally 0.5 ml of the appropriate cell suspension. Fourteen days later the mice were challenged intracerebrally with 0.025 ml of a suspension containing 50,000 viable cells of *B. pertussis* strain 18-323. This dose contained approximately 200 LD_{50}'s. Deaths were recorded for a period of 14 days.

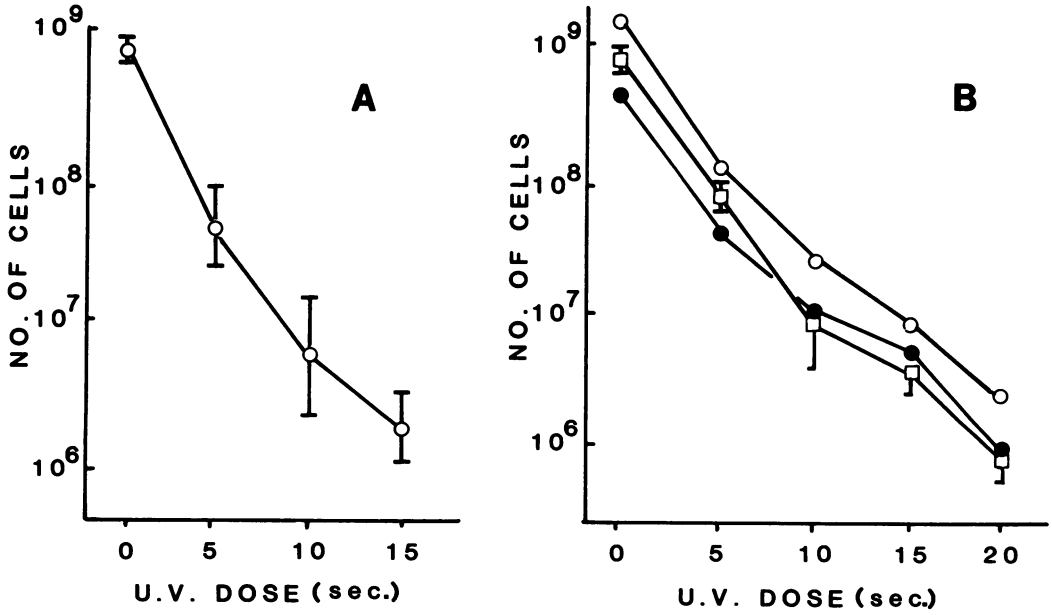

FIG. 1. (A) Survival of *B. pertussis* strain 18-323 after UV irradiation. Average of five different experiments. (B) Survival of various strains of *B. pertussis* after UV irradiation. Symbols: ○, parent; ●, FHA-producing strain; □, average results obtained with five mutants lacking ability to produce FHA.

TABLE 1. HA production by various strains of *B. pertussis*

Strain	HA titer (5×2^n)			
	Chicken	Horse	Sheep	Human (O)
18-323	2.9 ± 1.6	4	3	3
UV-1509	3.4 ± 2.3	3	2	ND[a]
UV-1529	3.7 ± 2.3	4	2	ND
UV-1541	4.2 ± 1.3	4	3	2
UV-1544	<1	<1	<1	<1
UV-1549	<1	<1	<1	<1
UV-1550	<1	<1	<1	<1
UV-1551	3.8 ± 1.6	4	2	4
UV-1555	4.2 ± 0.9	3	2	3
UV-1556	<1	<1	<1	<1
UV-1570	<1	<1	<1	<1
NKW	4.3 ± 0.6	6	5	3
Sakairi	<1	<1	<1	<1

[a] ND, Not done.

The dose of cells protecting 50% of the mice (PD_{50}) was then calculated by the method of Worcester and Wilson (25).

RESULTS

UV rays sensitivity of *B. pertussis*. Broth cultures (Cohen-Wheeler medium) of *B. pertussis* strain 18-323 were diluted in 1% Casamino Acids solution to contain 10^3 to 10^6 cells per ml and plated on BG agar. The plates were placed at a distance of 30 cm under a UV lamp and irradiated for various lengths of time. After irradiation, the plates were incubated 48 h at 36°C, and the colonies that developed were counted. Figure 1 shows the survival curves of *B. pertussis* in five different experiments. Under the conditions described, 15-s exposure to UV irradiation killed 99.7% of the cells. This exposure was used to irradiate cultures to induce mutants lacking the ability to produce FHA.

Isolation of mutants. Seventy colonies obtained from UV-irradiated cells were cultured on fresh BG agar and tested for their ability to produce FHA. The results are summarized in Table 1. The parent strain 18-323 and five other cultures obtained from UV-irradiated plates (UV-1509, UV-1529, UV-1541, UV-1551, and UV-1555) produced FHA when tested against chicken, horse, sheep, and human O group erythrocytes. Five strains (UV-1544, UV-1549, UV-1550, UV-1556, and UV-1570) failed to produce FHA. The mutant strains lacking the ability to produce FHA were stable even after 10 transfers on BG agar.

TABLE 2. Serological test on various strains of *B. pertussis*

Strain	HA	Agglutinogen type	Agglutinin titer (100×2^n)
18-323	+	1.2.4	4.9 ± 1.1
UV-1509	+	1.2.4	5.1 ± 1.1
UV-1529	+	1.2.4	5.0 ± 1.1
UV-1541	+	1.2.4	5.0 ± 0.9
UV-1544	−	1.2.4	5.1 ± 1.2
UV-1549	−	1	3.4 ± 1.8
UV-1550	−	1	3.8 ± 1.9
UV-1551	+	1.2.4	5.6 ± 1.0
UV-1555	+	1.2.4	5.6 ± 1.0
UV-1556	−	1	2.6 ± 1.6
UV-1570	−	1	2.3 ± 2.6
NKW	+	1	<1
Sakairi	−		<1

Biological assay. Each strain was tested for the production of agglutinogen factors 1 to 4 and for O antigen by agglutination tests with specific antisera (Table 2). The parent strain 18-323 and five FHA-producing strains were found to be serotype 1.2.4. On the other hand, five strains that lack the ability to produce FHA were found to be serotype 1. Only one mutant, UV-1544, was found to retain all the agglutinogen (1.2.4) of the parent strain. All strains used did not agglutinate in the presence of O antiserum (anti-Sakairi serum) at a dilution of 1/50, whereas the Sakairi cells were agglutinated by this antiserum diluted 1/3,200.

TABLE 3. Virulence test of various strains of *B. pertussis*

Strain	Agglutinogen type	HA	LD_{50}			
			Expt 1	Expt 2	Expt 3	Expt 4
18-323	1.2.4	+	2.50×10^4	2.51×10^4	3.15×10^4	5.00×10^4
UV-1509	1.2.4	+	4.88×10^5		1.58×10^5	
UV-1529	1.2.4	+	3.15×10^5	2.51×10^4		
UV-1541	1.2.4	+	1.58×10^5		1.26×10^5	
UV-1544	1.2.4	−		3.16×10^5	7.92×10^7	1.58×10^7
UV-1549	1	−		3.79×10^6	3.15×10^7	9.98×10^7
UV-1550	1	−	1.25×10^6	9.98×10^6	3.79×10^7	5.00×10^7
UV-1551	1.2.4	+	3.15×10^4	2.00×10^4		1.26×10^4
UV-1555	1.2.4	+	7.91×10^4	2.51×10^4		
UV-1556	1	−	1.99×10^5	7.93×10^6		9.98×10^5
UV-1570	1	−			6.29×10^6	
NKW	1	+		9.98×10^6	6.29×10^7	1.26×10^7

TABLE 4. Leukocytosis-promoting activity of various strains of *B. pertussis*

Strain	Agglutinogen type	HA	Leukocyte count ratio[a]				
			Expt 1	Expt 2	Expt 3	Expt 4	Avg
18-323	1.2.4	+	4.59	3.79	2.88	2.16	3.75 ± 0.86
UV-1509	1.2.4	+	3.75		5.05		4.40 ± 0.92
UV-1529	1.2.4	+		4.93		2.93	3.93 ± 1.41
UV-1541	1.2.4	+	3.20		5.24		4.22 ± 1.44
UV-1544	1.2.4	−	2.86	4.76	3.08	1.93	3.57 ± 1.04
UV-1549	1	−	2.77	3.79	5.27	1.53	3.94 ± 1.26
UV-1550	1	−		2.98	3.65		3.27 ± 0.54
UV-1551	1.2.4	+			4.16	2.52	3.34 ± 1.16
UV-1555	1.2.4	+	2.83	4.35		1.98	3.59 ± 1.07
UV-1556	1	−		4.44		2.18	3.31 ± 1.59
UV-1570	1	−	1.70	1.84		2.02	1.77 ± 0.10
NKW	1	+			7.63	2.13	4.88 ± 3.89

[a] Leukocyte count ratio = the mean number of leukocytes 3 days after inoculation/the mean number of leukocytes before inoculation.

Dermonecrotic toxin production was observed in the five mutants that did not produce FHA. Positive dermonecrotic reactions were obtained with 100 million cells, but not with 10 million cells given intradermally to guinea pigs. The necrotic lesions produced were typical for dermonecrotic reactions, and they were not produced by cells heated at 56°C for 30 min (14).

The results of the virulence tests of various strains are given in Table 3. The LD_{50} for the parent strain and the FHA-producing strains was from 10^4 to 10^5 cells, whereas the LD_{50} for the mutant strains lacking the ability to produce FHA was from 10 to 1,000 times greater. The NKW strain produced FHA and had low virulence (LD_{50} around 10^7 cells).

Table 4 gives the results of the leukocytosis-promoting tests. In this table the results are given as the ratio of leukocyte count in blood 3 days after the injection of *B. pertussis* cells over the leukocyte count taken before the animals had received the bacterial suspension. It is clear that all of the suspensions at a dose of 10^9 cells increased the leukocyte count by a factor of from 2 to 4 with only one exception, strain UV-1570, which increased the count by a factor of 1.77. No clear correlation was found between FHA production and leukocytosis-promoting activity.

The results of histamine-sensitizing activity are given in Table 5. No correlation was found between FHA production and histamine sensitization.

Mouse protection tests were carried out at three different doses of each strain (1,500, 300, and 60 million per mouse). The results are given in Table 6. The PD_{50} of each strain was found to be 10^8 to 10^9 cells per mouse for all strains. Thus, no correlation was found between FHA activity and mouse protective activity.

DISCUSSION

Keogh et al. (9, 10) reported that saline suspensions of the *B. pertussis* cells, as well as cell-free supernatant fluids of broth cultures of these bacteria, were able to agglutinate erythrocytes from various animal species. Most freshly isolat-

TABLE 5. Histamine-sensitizing activity of various strains of *B. pertussis*

Strain	Agglutinogen type	HA	Histamine-sensitizing activity (deaths/total)					
			Expt 1	Expt 2	Expt 3	Expt 4	Total	% Dead
18-323	1.2.4	+	4/5	4/5	3/5	1/5	12/20	60
UV-1509	1.2.4	+	3/5		2/5		5/10	50
UV-1529	1.2.4	+		2/5		0/5	2/10	20
UV-1541	1.2.4	+	4/5		4/5		8/10	80
UV-1544	1.2.4	−	3/5	4/5	0/5	3/5	10/20	50
UV-1549	1	−	4/5	4/5	3/5	4/5	15/20	75
UV-1550	1	−		2/5	3/5		5/10	50
UV-1551	1.2.4	+			2/5	4/5	6/10	60
UV-1555	1.2.4	+	0/5	5/5		1/5	6/15	40
UV-1556	1	−		5/5		3/5	8/10	80
UV-1570	1	−	0/5	0/5		0/5	0/15	0
NKW	1	+			4/5	3/5	7/10	70

TABLE 6. Mouse protective activity of various strains of *B. pertussis*

Strain	Agglutinogen serotype	HA	PD_{50}	
			Expt 1	Expt 2
18-323	1.2.4	+	2.60×10^8	1.65×10^9
UV-1509	1.2.4	+	1.71×10^8	1.32×10^8
UV-1529	1.2.4	+	2.07×10^8	7.40×10^8
UV-1541	1.2.4	+	1.30×10^8	4.01×10^8
UV-1544	1.2.4	−	ND[a]	ND
UV-1549	1	−	ND	3.47×10^9
UV-1550	1	−	2.77×10^8	5.44×10^8
UV-1551	1.2.4	+	1.88×10^8	4.78×10^8
UV-1555	1.2.4	+	2.52×10^8	4.35×10^8
UV-1556	1	−	7.39×10^8	9.72×10^8
UV-1570	1	−	ND	1.97×10^9

[a] ND, Not done.

ed strains of *B. pertussis* produced HA (12, 21). Kasuga et al. (7) reported that all phase I strains agglutinated horse erythrocytes, and many of them also agglutinated erythrocytes from oxes, chickens, and humans. The phase III and rough strains did not agglutinate any of these erythrocytes. In the present studies, strain 18-323 was able to agglutinate chicken erythrocytes, but the titers were lower than those obtained with other phase I strains (unpublished data).

By UV irradiation we were able to obtain five mutants of *B. pertussis* 18-323 that lacked the ability to produce FHA. The mutants appeared to be stable, since after 10 passages on BG agar they were still incapable of producing FHA. A comparison of the biological activities of these mutants with the parent strain revealed that all had leukocytosis-promoting, histamine-sensitizing, and mouse-protecting activities. Since five of these strains lacked the ability to produce FHA, it appears that FHA is not required for those biological activities. This reinforces the results of Nakase and Doi (15) and Munoz et al. (13), who found that FHA by itself is not capable of protecting or of inducing histamine sensitization and leukocytosis in mice.

Among the mutants lacking the ability to produce FHA we found that four contained only agglutinogen 1 and one strain retained the three (1.2.4) agglutinogens found in the parent strain. The significance of this finding is not clear at present, except that agglutinogens 2 and 4 are not associated with the production of pertussigen, the substance responsible, according to Munoz and Bergman (14), for mouse protection, leukocytosis, and histamine sensitization.

One observation that deserves mentioning is the fact that the virulence of the mutant cultures lacking the ability to produce FHA was lower than that of the parent strain (Table 3). It required from 10 to 1,000 times more cells of these mutant cultures than of those of the parent culture to kill mice by the intracerebral route. This finding may indicate that FHA plays a role in the virulence of *B. pertussis*, but FHA is not the only factor involved, since the NKW strain that produced FHA had a virulence as low as the mutant strains that lacked the ability to produce FHA. More work is needed to find the exact role of FHA and other antigens in the virulence of *B. pertussis*.

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