Cross-Reacting Serum Opsonins in Patients with Meningococcal Disease

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We have examined the opsonic activity of sera from patients with *Neisseria meningitidis* (B:15:P1.16) infections against different meningococcal strains, using flow cytometry and luminol-enhanced chemiluminescence. A marked increase in the phagocytosis of ethanol-fixed meningococcal strains of different serogroups, serotypes, and serosubtypes was demonstrated in the presence of convalescence sera compared with acute sera. Convalescence sera also caused a significant increase of leukocyte oxidative metabolism during phagocytosis, as measured by luminol-enhanced chemiluminescence. The sera contained a broad range of opsonins cross-reacting with serogroup A, B, C, W-135, and Y meningococci of different serotypes and serosubtypes, indicating that the cross-reacting opsonins recognized surface epitopes other than those determined by current serotyping schemes.

Patients with meningococcal disease develop serum antibodies against different capsular and outer membrane antigens. Although these antibodies confer opsonic (10, 20, 23) as well as bactericidal (7) function, the susceptibility to systemic disease has been related to the lack of bactericidal antibodies to pathogenic strains of *Neisseria meningitidis* (7). However, more recent studies have indicated that opsonic antibodies may play a role in the defense against group B meningococcal disease (10, 18, 22).

Antibodies directed against meningococcal outer membrane antigens can cross-react with outer membrane antigens from heterologous strains. Cross-reacting antibodies have been detected against meningococcal minor outer membrane proteins, major outer membrane proteins (class 2 and class 3), lipopolysaccharide, and pili (15, 17). Poolman et al. (17) have shown in nonfunctional assays that convalescence sera from patients with meningococcal disease show significant cross-reactivity against outer membrane complexes from heterologous meningococcal strains. Cross-reacting bactericidal antibodies have been demonstrated in patients with meningococcal disease (12) and in vaccinees immunized with meningococcal group B outer membrane vesicles (11). There is evidence of cross-reacting serum opsonins in convalescence sera from patients with meningococcal disease (10, 13), but little is known about the extent of this opsonic cross-reactivity.

The ongoing epidemic of meningococcal group B disease in Norway has been dominated by serotype 15 (6). Since opsonophagocytosis of meningococci seems to be of importance in the defense against group B meningococcal disease (10, 18, 22), we have examined the opsonophagocytosis of different meningococcal strains with sera from patients with *N. meningitidis* (B:15:P1.16) infections to assess cross-reacting opsonic activity. For this purpose, we used flow cytometry for the study of phagocytosis and chemiluminescence for the study of leukocyte oxidative metabolism during phagocytosis.

(Part of this work was presented at the 7th International Pathogenic Neisseria Conference in Berlin, Germany, 1990 [8].)

MATERIALS AND METHODS

Sera. Sera from seven patients (Table 1) with systemic infections due to *N. meningitidis* (B:15:P1.16) were obtained on admission to hospital (within 2 days from onset of symptoms) and 6 weeks later. Sera were also obtained from two patients on admission to hospital and 6, 26, and 52 weeks after the acute infection. All sera were immediately frozen and stored in aliquots at −70°C until use.

Materials. Fluorescein isothiocyanate (FITC), heme, luminol, and NADH were obtained from Sigma Chemical Co., St. Louis, Mo. Heart infusion broth was purchased from Difco Laboratories, Detroit, Mich., and supplemented with 1 g of DST agar (Oxoid, Basingstoke, England) per liter of broth. Brain heart infusion broth was purchased from GIBCO, Paisley, Scotland. Human serum albumin was from KabiVitrum, Stockholm, Sweden. Erythrocyte-lysing solution consisted of 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 10 mM EDTA (pH 7.4) (4).

Leukocytes. Heparinized (14 U/ml) blood from healthy, nonsmoking (19) volunteers diluted 1:10 in erythrocyte-lysing solution (4) was mixed continuously at 20°C until lysis (5 to 8 min). After being washed (500 × g for 5 min) with phosphate-buffered saline (PBS) supplemented with 0.5% human serum albumin, the remaining erythrocytes in the cell suspensions were lysed with 150 mM NH_4Cl during continuous mixing at 20°C for 5 min. The leukocytes were washed twice, and platelets were removed during the last washing step by centrifugation at 350 × g. The leukocytes were resuspended in Hanks' balanced salt solution (HBSS) containing 0.5% human serum albumin and adjusted to 1.25 × 10^7 nonlymphocytes (i.e., polymorphonuclear leukocytes and monocytes) per ml. Total and differential leukocyte counts were measured by using an S-plus III Coulter Counter (Coulter Electronics, Harpenden, England). The cell suspensions contained 26 to 45% mononuclear cells, and monocytes never accounted for more than 10% of the total

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TABLE 1. Clinical and laboratory parameters upon hospital admission of patients whose sera were included in this study[a]

Patient no.	Age (yr)	Sex[b]	Blood pressure (mm Hg)	Core temp (°C)	Neck/back rigidity[c]	Consciousness	Blood content (10⁹/liter) of:		CSF content of leukocytes (10⁶/liter)
							Leukocytes	Platelets	
1	22	F	100/70	38	(+)	Somnolent	20.4	178	54
2	45	F	140/85	40	+	Conscious	11.2	178	4,076
3	55	F	160/70	39.9	+	Somnolent	18.5	195	3,450
4	17	M	115/80	38	+	Soporous	18.8	172	4,750
5	18	F	140/80	39.4	+	Somnolent	21.8	371	1,115
6	28	M	ND[d]	<37	–	Soporous	22.4	98	—[e]
7	20	F	140/80	39.7	+	Somnolent	14.2	267	241

[a] All patients survived, and only one (patient 7) developed sequelae. Petechiae were present in all patients.
[b] F, female; M, male.
[c] (+), doubtful; +, present; –, absent.
[d] ND, not detectable.
[e] —, not done.

cell suspension. However, 20% monocytes in the final cell suspension did not influence the results (data not shown). The cell viability was ≥99% by trypan blue (0.1% [wt/vol] in PBS) exclusion.

Bacterial strains and culture conditions. The N. meningitidis strains used in this study were chosen according to the Norwegian meningococcal epidemiological data (6). The bacteria of different serogroups, serotypes, and serosubtypes (Table 2) were grown overnight on DST-blood agar in a 5% CO_2 atmosphere at 37°C, inoculated in heart infusion broth to an optical density of 0.4 at 620 nm, using a 10-mm light path, and grown to logarithmic growth phase in a shaking water bath at 37°C (3 to 4 h, interstrain variations). The bacteria were washed three times in ice-cold 0.9% NaCl (2,500 × g for 10 min at 4°C), resuspended in HBSS (pH 7.4), and adjusted to an optical density of 1.0, and CFU per milliliter was determined. The bacteria were not killed by this procedure.

Ethanol fixation of the bacteria. Suspensions of bacteria were centrifuged (2,500 × g for 10 min at 4°C), resuspended in an equal volume of ice-cold 70% ethanol, and kept on ice for 1 h. After three washes in ice-cold 0.9% NaCl, the bacteria intended for chemiluminescence assays were resuspended in HBSS (pH 7.4), counted in a counting chamber (Thoma, Karl Hecht, Sonderheim, Germany), adjusted to 5 × 10⁸ bacteria per ml, and stored in aliquots at −70°C. The ethanol-fixed and unfixed bacteria gave identical results by

serogroup agglutination. Previous studies (2, 9) and preliminary results demonstrated no significant differences between using live and ethanol-fixed microorganisms for our phagocytosis and chemiluminescence assays.

FITC labeling of the bacteria. Ethanol-fixed bacteria were labeled with FITC by stirring the bacteria for 30 min at 37°C in PBS containing 0.5 mg of FITC per ml. The bacteria were washed four times in PBS (2,500 × g for 10 min at 4°C), resuspended in HBSS (pH 7.4), counted by flow cytometry, adjusted to 5 × 10⁸ bacteria per ml, and stored in aliquots at −70°C (1, 3).

Flow cytometry assay. Bacterial suspensions (2.5 × 10⁷ bacteria in HBSS) and sera (final concentration of 5%) were mixed in plastic tubes by end-over-end rotation for 7.5 min at 37°C. Titration of sera showed that 5% was sufficient for maximal phagocytosis, and heat treatment of sera (56°C for 30 min) reduced the phagocytosis to 10 to 20% of the level obtained with non-heat-treated sera. After opsonization, 0.1 ml of the leukocyte suspension was added to give a ratio of 20 bacteria per nonlymphocyte. Phagocytosis was terminated after 7.5 min by adding 1 ml of ice-cold PBS supplemented with 0.02% EDTA. The suspensions were analyzed by a Coulter EPICS V flow cytometer with a 488-nm argon laser, interfaced to a MDADS computer (Coulter Electronics Ltd., Luton, England) (1, 3). A 530-nm band pass filter was used for measurements of FITC fluorescence.

Flow cytometry parameters of phagocytosis. FITC-labeled

TABLE 2. N. meningitidis strains used

Strain designation/ year of isolation	Serogroup:serotype:serosubtype[a]	Outer membrane protein class	Source (reference)
44/76[b]	B:15:P1.16	3	Patient isolate, Norway (26)
41040/83[c]	B:15:P1.16	3	Patient isolate, Norway
18283/85[c]	B:15:P1.16	3	Patient isolate, Norway
52085/86[c]	C:15:P1.16	3	Patient isolate, Norway
8141/81[b,c]	C:2a:P1.2	2	Patient isolate, Norway
138 I	C:2a:P1.2	2	Patient isolate (26)
M 986	B:2a:P1.2	2	Patient isolate (26)
M 986 NCV	–:2a:P1.2	2	Nonencapsulated variant of M 986
8047	B:2b:P1.2	2	Patient isolate (26)
A 853/83[c]	A:4	3	Patient isolate, The Gambia
Y	Y:NT	Not known	Vaccine strain (14)
W-135	W-135:NT:P1.16	Not known	Vaccine strain (14)

[a] In accordance with the proposed serotyping scheme by Frasch et al. (5).
[b] Isolated from a patient who died during the course of the meningococcal disease.
[c] More recently isolated patient strain.

free extracellular bacteria, nonphagocytes, and phagocytes were discriminated and quantified by combined measurements of fluorescence and light scatter (1, 3). The number of bacteria per phagocyte was defined as the difference between the initial and final counts of extracellular bacteria divided by the number of phagocytes (1, 3).

Chemiluminescence. The sensitivity of the chemiluminescence method as used by Halstensen and Haneberg (9), was enhanced by using 1 μM (final concentration) luminol as an amplifier. The bacteria (2.5 × 10^7 bacteria in HBSS) were incubated with 25 μl of serum (acute or convalescence) during agitation for 7.5 min at 37°C. Increasing the concentration of sera did not affect the results. Then 0.1 ml of leukocyte suspension was added to the reaction mixture, giving a ratio of 20 bacteria per nonlymphocyte and a total volume of 0.5 ml. The chemiluminescence production (millivolt · seconds) during phagocytosis was measured for 7.5 min by an automatic photoluminometer (LKB Wallac Luminometer 1251; Wallac, Turku, Finland) with a built-in temperature and mixing control system. The results are expressed as integrals of the light emitted during phagocytosis (millivolt · seconds). An internal control of a standard human serum and reaction mixtures without sera were included in each experiment. Heat treatment of the sera (56°C, 30 min) resulted in a reduction of the chemiluminescence to 10 to 20% of the level obtained with non-heat-treated sera.

Microscopy. Flow cytometry reaction mixtures were examined by combined light and fluorescence microscopy, using an Orthoplan microscope equipped with a Ploemopak Fluorescence Vertical Illuminator (Leitz, Wetzlar, Germany). The number of phagocyte-associated bacteria was determined for 100 phagocytes in each suspension.

Controls. Control strains used were *Haemophilus influenzae* type b (strain NCTC 8468 and a patient isolate [165/86] from the National Institute of Public Health, Oslo, Norway), *Streptococcus pneumoniae* type 1 N (reference strain from the National Institute of Sera, Copenhagen, Denmark, and a patient isolate [403/90] from the National Institute of Public Health, Norway), and *Staphylococcus epidermidis* (a patient isolate [41608/80] from Haukeland Hospital, Bergen, Norway). The pneumococci were grown overnight on DST-blood agar, inoculated in brain heart infusion broth to an optical density of approximately 0.4, and grown to logarithmic growth phase in a shaking water bath at 37°C. The *H. influenzae* strains were grown overnight on DST-blood agar supplemented with heme (10 mg/liter) and NADH (10 mg/liter), inoculated in brain heart infusion broth supplied with heme (10 mg/liter) and NADH (10 mg/liter), and grown to logarithmic phase as for the pneumococci. *S. epidermidis* was cultured under the same conditions as were the meningococci. The control strains were ethanol fixed and FITC labeled as for the meningococci.

Statistical methods. Nonparametric statistics were used, utilizing SPSS/PC+ V3.0 statistical software, and the continuous variables were expressed as median values. The Wilcoxon signed rank test was used to determine the statistical significance of differences in opsonic activity observed between convalescence and acute sera, and the Mann-Whitney test was used to assess the statistical significance of differences between independent samples. A two-sided probability value of <0.05 was considered statistically significant (21).

RESULTS

Phagocytosis. The opsonic activity of convalescence sera (6 weeks) from patients with *N. meningitidis* (B:15:P1.16) infections was significantly higher than that of acute sera when the samples were incubated with meningococci of various serogroups, serotypes, and serosubtypes ($P < 0.05$ for all strains tested, using the Wilcoxon signed rank test) (Fig. 1). This increase in opsonic activity as measured by the number of bacteria per phagocyte was significant regardless of the opsonic activity of the acute sera. Thus, the convalescence sera contained a wide range of opsonins reacting with serogroup A, B, C, W-135, and Y meningococci of different serotypes and serosubtypes.

The opsonic activity of the sera varied against strains of the same serotype and serosubtype as the infecting strains, which were all of serotype B:15:P1.16. The number of bacteria per phagocyte was significantly higher for the laboratory strain (44/76) than for the recently isolated patient strains ($P < 0.05$, using the Mann-Whitney test) (Fig. 1). However, the increase in opsonic activity was greater for the newer patient isolates ($P < 0.05$). The same trend was found for the two C:2a:P1.2 strains tested; the laboratory strain 138 I ($P < 0.05$) was phagocytized to a greater degree than was the recently isolated patient strain with both acute and convalescence sera. However, the increase in opsonic activity was greater for the patient isolate than for strain 138 I. The laboratory strain M 986 (B:2a:P1.2) and its nonencapsulated variant, both of which were phagocytized to a great extent with acute sera, were both phagocytized to the same extent with convalescence sera.

Sera from two meningococcal disease patients (B:15:P1.16) collected 6 and 12 months after hospital admission still contained increased levels of opsonins cross-reacting with different meningococcal strains ($P < 0.05$, using the Wilcoxon rank sum test) (Fig. 2). The number of bacteria per phagocyte as measured by flow cytometry correlated well and was of the same magnitude as that determined by combined light and fluorescence microscopy (data not shown). Moreover, flow cytometry and fluorescence microscopy demonstrated that meningococci were found in each phagocyte examined. Meningococci were not phagocytized in the absence of serum. There was no increase in the phagocytosis of *H. influenzae* type b, *S. pneumoniae* type 1 N, and *S. epidermidis* when the bacteria were incubated with convalescence sera compared with acute sera from patients with meningococcal disease (data not shown).

Oxidative burst. All meningococcal strains tested induced a significantly greater chemiluminescence response with convalescence sera than with acute sera ($P < 0.05$ for all strains tested, using the Wilcoxon signed rank test), demonstrating the presence of a broad range of opsonins cross-reacting with all meningococcal strains (Fig. 3). The oxidative response during phagocytosis as measured by chemiluminescence varied against strains of the same serotype and serosubtype as the infecting strains (B:15:P1.16), and the increase in the oxidative burst was greater when the newer isolated strains were used than when the laboratory strain was used ($P < 0.05$, using the Mann-Whitney test). The increase in the oxidative burst during phagocytosis was also greater with the newer patient isolate than with the laboratory strain of serotype C:2a:P1.2 tested ($P < 0.05$). The increased chemiluminescence response observed with convalescence sera (6 weeks) compared with acute sera was still seen with sera obtained from two patients 6 and 12 months after the acute infection (data not shown).
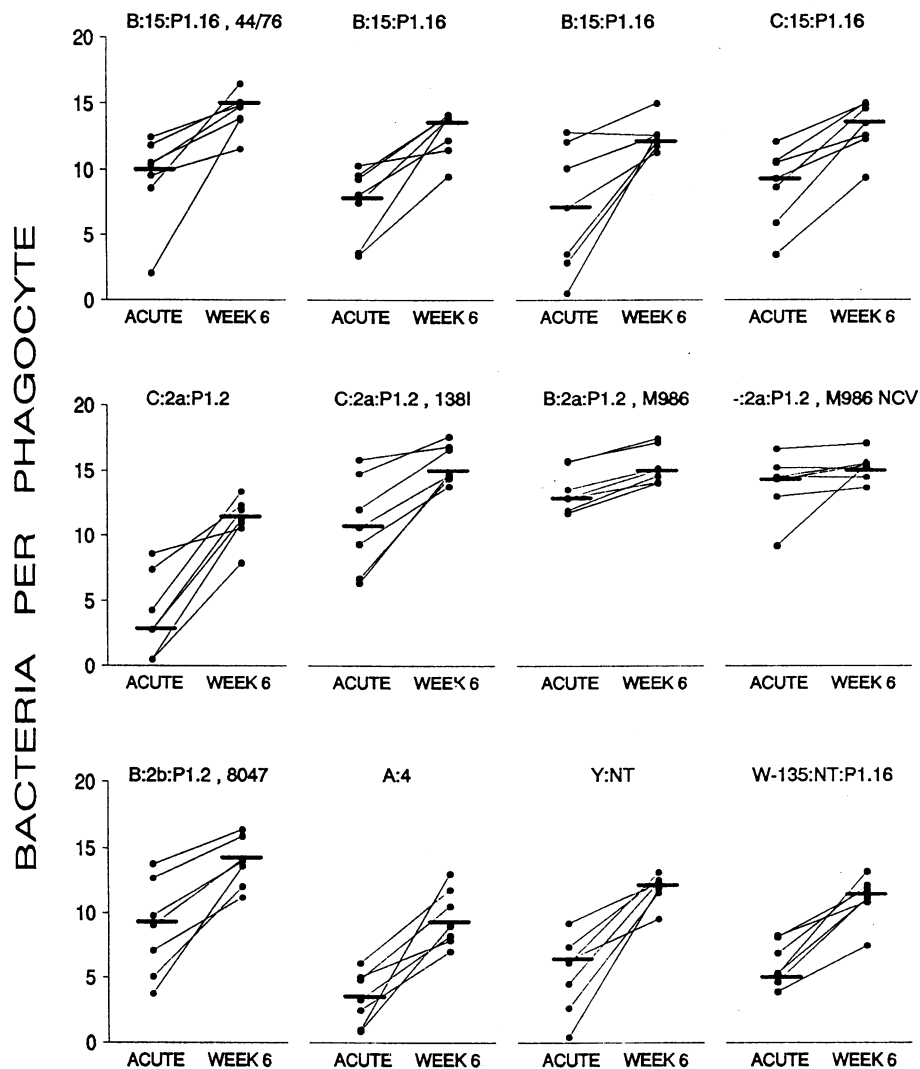
FIG. 1. Phagocytosis (number of bacteria per phagocyte) with acute and convalescence sera from patients with systemic meningococcal disease due to B:15:P1.16 strains. Each panel illustrates the results obtained with use of the seven different pairs of sera against one meningococcal strain. Each point represents the mean of two parallel examinations. The results with acute and convalescence sera from each patient are connected with a line. Horizontal lines indicate median values.

A significantly lower chemiluminescence response was observed with use of the serotype 2a or 2b strains containing class 2 outer membrane protein than with use of serotype 15 strains (class 3) regardless of the serogroup ($P < 0.05$, using the Mann-Whitney test). The oxidative response during phagocytosis of the nonencapsulated variant of the B:2a:P1.2 strain was twice as high as that of the encapsulated parent strain.

There was minimal chemiluminescence activity in the absence of serum. No increase in the polymorphonuclear leukocyte oxidative metabolism was demonstrated during the phagocytosis of different strains of *H. influenzae* type b, *S. pneumoniae* type 1, and *S. epidermidis* tested with convalescence sera compared with acute sera from patients with meningococcal disease (data not shown).

DISCUSSION

Cross-reacting antibodies against meningococcal outer membrane complexes have been demonstrated in convales-
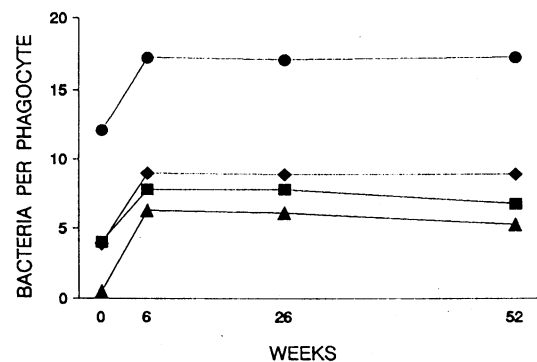

FIG. 2. Phagocytosis with acute (0 weeks) and convalescence (6, 26, and 52 weeks) sera from one patient with systemic meningococcal infection (B:15:P1.16) and four meningococcal strains of different serogroups, serotypes, and serosubtypes. Symbols: ■, B:15: P1.16, 44/76; ▲, C:15:P1.16; ●, C:2a:P1.2, 138 I; ◆, A:4.
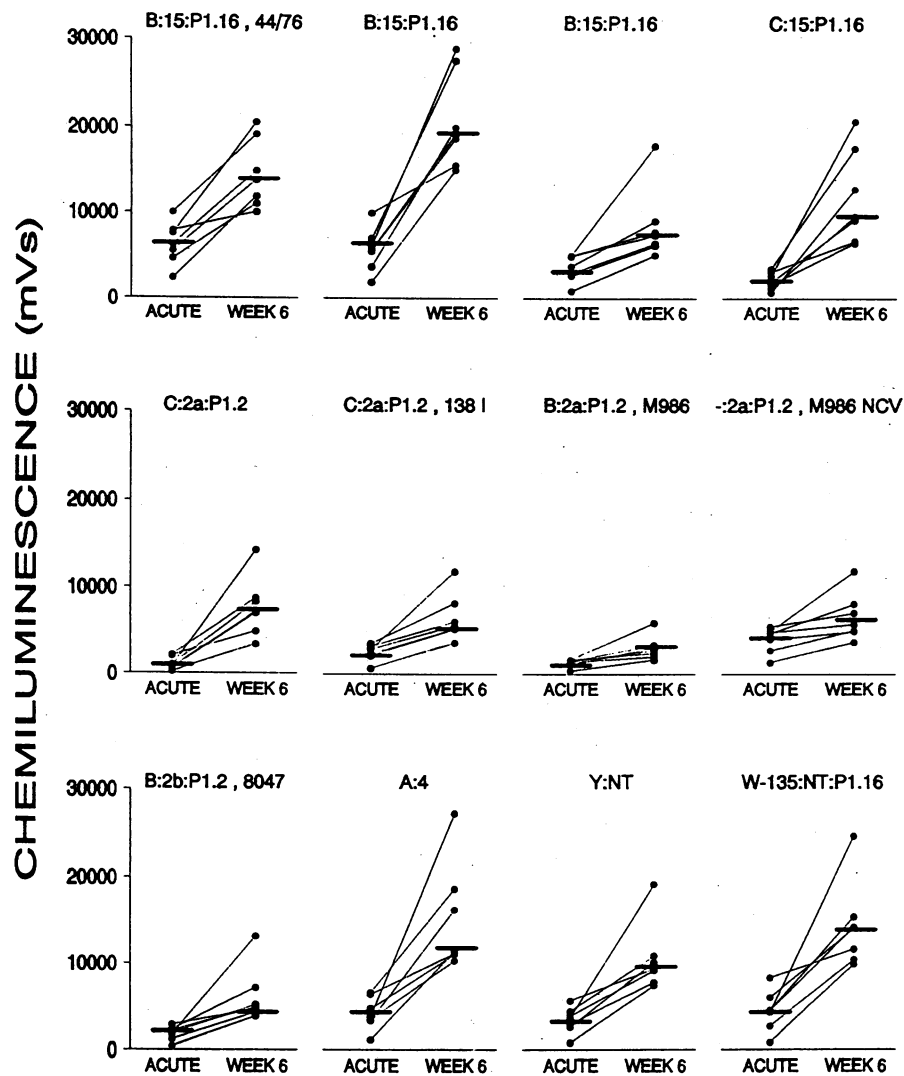
FIG. 3. Oxidative burst. Luminol-enhanced chemiluminescence of polymorphonuclear leukocytes stimulated with meningococci opsonized with acute and convalescence sera from patients with systemic disease due to *N. meningitidis* (B:15:P1.16). Each panel illustrates the results obtained with use of the seven different pairs of sera against one meningococcal strain. Each point represents the mean of two parallel examinations. The results for acute and convalescence sera from the same patient are connected with a line. Horizontal lines indicate median values. mVs, millivolt · seconds.

cence sera from patients with meningococcal group B disease and in sera from vaccinees immunized with meningococcal group B outer membrane vesicles (10–13, 15, 17). Such cross-reacting antibodies have been shown in quantitative, nonfunctional assays (15, 17) as well as in bactericidal assays (11, 12). However, little is known about the extent of opsonic cross-reactivity in convalescence sera toward meningococci of other serogroups, serotypes, and serosubtypes than those of the infecting strain (10, 13). In the present study, we have examined the presence of cross-reacting opsonins in convalescence sera from patients with *N. meningitidis* (B:15:P1.16) infections. The opsonophagocytosis was determined by flow cytometry, and the leukocyte oxidative burst during phagocytosis was quantitated by luminol-enhanced chemiluminescence. Cross-reacting serum opsonins were demonstrated against serogroup A, B, C, W-135, and Y meningococci of all serotypes and serosubtypes tested. Furthermore, the variations in the opsonophagocytosis of meningococci did not follow serotype and sero-

subtype patterns, indicating that opsonins cross-react with epitopes other than those defined by serotyping. This is in accordance with the results of previous studies using enzyme-linked immunosorbent assay and radioimmunoassay techniques (15, 17).

In this study, laboratory strains of meningococci were more easily phagocytized than were recently isolated patient strains of the same serogroup, serotype, and serosubtype. Most likely, strains become attenuated and more susceptible to phagocyte ingestion during continued laboratory passages. In functional assays, it may thus be wise to include recently isolated patient strains in addition to well-characterized laboratory strains.

Usually, encapsulated bacteria are more resistant to phagocytosis than are nonencapsulated strains (24). In this study, however, the B:2a:P1.2 strain (M 986) and its nonencapsulated variant (M 986 NCV) were phagocytized to approximately the same extent, and the phagocytosis of each strain in the presence of both acute and convalescence sera

was more pronounced than was that of any other strain tested. The reasons for the similar phagocytosis of these two strains are not known, but the serogroup B capsule is poorly immunogenic (16, 25), and antibodies to subcapsular epitopes seem to be more important for the phagocytosis of these strains than are capsular antibodies.

Our study indicates that the number of bacteria phagocytized does not correspond directly to the leukocyte oxidative metabolism during phagocytosis as measured by luminol-enhanced chemiluminescence. This cannot be accounted for by differences in the reaction mixtures, since leukocytes from one donor and meningococci from a single culture were used for parallel flow cytometry and chemiluminescence assays on the same day. Our results also suggest that the strains with outer membrane protein class 3 (serotypes 15 and 4) elicit greater chemiluminescence than do the class 2 strains (serotypes 2a and 2b). The reason for this finding is not known.

In a previous study (10), high levels of opsonic antibodies against N. meningitidis (44/76, B:15:P1.16) were demonstrated in sera collected from patients with meningococcal disease up to 5 years after the acute illness. In this study, significantly higher opsonic activity was demonstrated in sera collected up to 12 months after the acute illness than in acute sera, and the convalescence sera contained opsonins cross-reacting with meningococci of different serogroups, serotypes, and serosubtypes. This finding indicates that cross-reacting serum opsonins persist for a long time after the acute illness.

This study demonstrates that meningococcal infection with serogroup B:15:P1.16 strains induces cross-reactive serum opsonins reacting with strains of N. meningitidis of similar and different serogroups, serotypes, and serosubtypes. Capsular antibodies do not appear to contribute significantly to cross-reactive opsonization. Since sera from patients with group B meningococcal disease contained opsonins toward non-group B meningococcal strains, these antibodies probably recognize epitopes on the major outer membrane proteins or on other outer membrane antigens present on all strains tested, different from the epitopes recognized by the monoclonal antibodies used in current serotyping schemes. Future studies are in progress to discern which meningococcal components induce cross-reactive opsonins. Such data may lead to the selection of meningococcal vaccine components that induce potentially protective cross-reactive opsonins.

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