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Serum Bactericidal Effect on *Pseudomonas aeruginosa* Isolates from Cystic Fibrosis Patients

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The bactericidal activity against *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients was determined in a 10% concentration of normal serum or autologous cystic fibrosis serum. Of the 167 strains tested, 77 (46%) were sensitive (>95% killed) in normal serum. Mucoid strains were more frequently sensitive than nonmucoid strains. Twenty-three sensitive strains tested in ethyleneglycoltetraacetic acid-chelated serum were resistant (<10% killed), suggesting only classical pathway activation. Absorption of cystic fibrosis serum with the autologous *P. aeruginosa* strain resulted in decreased killing by that serum. All sera, including the chelated and absorbed sera, had comparable total hemolytic complement levels. Patients in poor clinical condition (5 out of 12), in contrast to patients in good or moderate condition (1 out of 30), were more likely to have *P. aeruginosa* strains that were serum resistant in autologous serum but sensitive in normal serum. Sera from these five patients in poor clinical condition were capable of killing heterologous *P. aeruginosa* strains. These results suggest the presence of a protective or "blocking" activity in serum from some patients in poor clinical condition. This association of a blocking activity with clinical condition may signal a transition point in the progression of cystic fibrosis lung disease and thus may be another contributory factor in the failure of the cystic fibrosis host to control infection.

Chronic pulmonary infection with *Pseudomonas aeruginosa* is a major factor in the morbidity and mortality associated with cystic fibrosis (CF). Eradication of this opportunistic pathogen by antibiotic therapy is virtually impossible, and clearance by mucociliary transport (2) and phagocytosis by the alveolar macrophage (14, 16) may be compromised in many patients. Humoral antibody response to *P. aeruginosa* is normal or elevated in most CF patients (9, 10), and systemic infection is rare (20). The role of serum bactericidal activity in CF patients as a host defense mechanism against the infecting *P. aeruginosa* has not been clearly established. CF strains of *Pseudomonas* are often more sensitive than one-CF strains to the bactericidal action of both normal and CF serum (7), but no reason for such increased sensitivity has been found.

The role of complement in pulmonary defense has not been clearly established. Complement components have been demonstrated in the lung (12), and the importance of complement in the opsonization of some strains of *P. aeruginosa* has been shown (11). Serum has also been implicated in direct killing of *P. aeruginosa* (21). Whether this killing activity is the result of classical pathway activity with antibody or alternate pathway activity without antibody or both has not been investigated. To differentiate between the pathways involved in the killing of CF *P. aeruginosa* strains, normal human serum chelated with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA) was utilized in our assay. Chelation of serum with 10 mM EGTA and 10 mM $MgCl_2$ blocks the classical pathway through binding of necessary Ca^{2+} ions, but allows activation of the alternate pathway, which requires Mg^{2+} ions but not Ca^{2+} ions (4). In addition, CF serum absorbed with *P. aeruginosa* and hypogammaglobulinemic serum were tested in this assay to determine the antibody requirement necessary for killing.

The aim of the present study was to characterize the parameters of the CF patient and his or her infecting *P. aeruginosa* strains that may correlate with the serum sensitivity of that strain. The parameters investigated included: (i) patient clinical condition, (ii) serum agglutinating antibody titer, (iii) serotype of *P. aeruginosa* strains, and (iv) morphological variety of *P. aeruginosa* strains.

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MATERIALS AND METHODS

Bacterial cultures. Sputum or, occasionally, deep-throat after-cough specimens were obtained from CF patients. *P. aeruginosa* was identified by Gram stain, oxidase reaction, pigment production and, if necessary, oxidative use of glucose. Isolates were classified into six morphological varieties (15): classic (smooth), rough, mucoid, gelatinous, dwarf, and enterobacter (smooth-rough) (18). Pure mucoid strains often yielded spontaneous nonmucoid revertant colonies after growth in broth or subculture to Mueller-Hinton agar (MHA; Difco Laboratories, Detroit, Mich.) or both. Revertant colonies were isolated, serotyped, and tested for serum sensitivity.

Serotyping of all isolates was carried out by the same individual, using the Homma and Difco typing systems as previously described (1).

Serum source. Blood from both normal volunteers and CF patients (not receiving antibiotic therapy) was obtained by venipuncture, allowed to clot for 1 h, and the serum was stored at −70° C until used. Total hemolytic complement levels were determined by radial diffusion plates (Quantiplate; Kallestad Laboratories, Chaska, Minn.). Hypogammaglobulinemic serum was obtained from a patient with agammaglobulinemia 24 h after transfusion. Serum immunoglobulin levels from this patient were as follows: IgG, 64 mg/dl; IgM, 9.8 mg/dl; IgA, 14 mg/dl. Serum chelated with 0.2 M EGTA (Sigma Chemical Co., St. Louis, Mo.) was prepared by the method of Fine (5).

The antibody titer of the serum samples against the bacteria used in the sensitivity assay was determined by slide agglutination as previously described (14).

Serum absorption with bacteria. A bacterial suspension of approximately 10^{10} organisms/ml in Hanks balanced salt solution was used for serum absorption. A 2:1 (vol/vol) suspension of serum to bacteria was incubated at 4°C for 60 min and centrifuged, and the absorption was repeated. After the second absorption, the absorbed serum was filter sterilized and used the same day.

Serum sensitivity assay. The serum sensitivity assay employed was modified after Young and Armstrong (21). Bacterial cultures were grown overnight with aeration in 10 ml of MH broth. Cultures were centrifuged at 10,000 rpm for 10 min and washed twice with DGVB (dextrose gelatin Veronal buffer; Flow Laboratories, Inc., McLean, Va.). Bacterial pellets were suspended in 5 ml of DGVB, adjusted to an optical density at 520 nm of 0.2 on a Spectronic 710 (Bausch & Lomb, Inc., Rochester, N.Y.) and diluted 10^{-3} to give a bacterial suspension containing 1×10^4 to 2×10^4 bacteria/ml. The assay tube consisted of 0.8 ml of DGVB, 0.1 ml of serum, and 0.1 ml of the bacterial suspension. Control tubes with heated serum (56°C for 30 min) were included for each bacterial strain, and *P. aeruginosa* and *Escherichia coli* C (ATCC 13706) strains of known sensitivity were tested with each serum. *E. coli* C is sensitive to the bactericidal activity of normal human serum and normal serum chelated with $MgCl_2$-EGTA (4). Assay tubes were incubated for 60 min at 37°C. Samples of 0.1 ml from undiluted and 10-fold dilutions were plated at T_0 and T_{60} onto MHA plates and incubated 18 to 24 h before scoring. Classification of serum sensitivity was as follows: sensitive (S), >95% killing; intermediate sensitive (IS), 50 to 95% killing; intermediate resistant (IR), 10 to 50% killing; resistant (R), <10% killing.

The intermediate category was divided into intermediate sensitive and intermediate resistant to clearly delineate between strains which were 50 to 95% sensitive and strains which had only a moderate amount of killing (10 to 50%). Few strains fell in the 50 to 70% killed range, so that a single intermediate category which included strains ranging in sensitivity from 10 to 95% did not accurately represent the observations we had made. Standard deviation of the assay is ±10% within the 10 to 95% killing range, i.e., the IS and IR categories, and ±3% in the S and R categories.

Patient evaluation. Clinical evaluation was based on a modification (3) of the Shwachman et al. (13) scoring system, in which a maximum of 25 points is awarded for each of four categories: chest roentgenogram, general activity, physical examination, and nutrition for a maximum (best possible) total score of 100 points. Patients were classified in consultation with their physician into three categories as follows: A, good clinical condition (total score, 75 to 100); B, moderate clinical condition (total score, 60 to 74); C, poor clinical condition (total score, >60).

RESULTS

Serum sensitivity in multiple-sterum samples. The bactericidal activity of normal, autologous CF and heterologous CF serum against *P. aeruginosa* isolates from CF patients was compared (Fig. 1). The percent sensitive in autologous serum (37%) is slightly decreased as compared to normal (46%) or heterologous CF serum (45%). All serum samples used in this study had comparable total hemolytic complement levels (all of the samples had levels above 70 CH_{100} [100% hemolytic complement] U/ml). Agglutinating antibody titer in CF and normal serum against the autologous CF strain was determined. As expected, titers were higher in CF serum, but did not appear to correlate with serum sensitivity. To further investigate this observation of apparent decreased sensitivity in autologous serum, the bactericidal activity of serum was correlated with the clinical condition of the patient from whom it was obtained (Table 1). No strain from the 20 patients in good clinical condition was more resistant in autologous serum than in normal serum. Only one patient (no. 25) of 10 (10%) in moderate condition had an isolate with greater resistance in autologous serum. In contrast, 5 of 12 (42%) patients in poor clinical condition had strains resistant in autologous but sensitive in normal serum. These results suggest that a difference exists between patients in poor condition and those in moderate or good condition with regards to the sensitivity of their strains in autologous serum. Chi-square (df = 1) comparison of patients in good or moderate condition with patients in poor condition

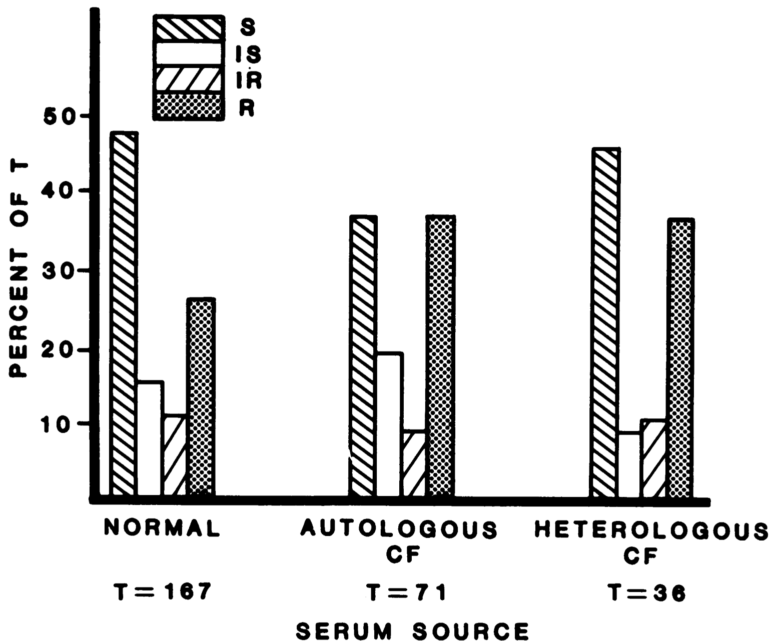

FIG. 1. *Comparison of the bactericidal activity of normal and autologous and heterologous CF serum against P. aeruginosa isolates (T, total number of strains).*

is significant. Sera from the patients in poor clinical condition were able to kill other sensitive strains of *P. aeruginosa* and *E. coli*. Addition of this "blocking" serum to normal serum in serial dilutions demonstrated that it could block bactericidal activity of normal serum against the autologous strain (Table 2). Further, this blocking activity may be quantitatively different since greater dilutions were necessary with the serum of patient no. 38 than with that of patient no. 31.

Serum sensitivity of subclones. Subclones of strains of *P. aeruginosa* previously determined to be S, IS, IR, or R were tested for their serum sensitivity to determine whether heterogeneity existed within a strain. All subclones yielded the same sensitivity classification as the parent strain. For intermediate strains, subclones were chosen from the strain before the sensitivity assay (possible S subclone) and from the serum T_{60} plate (R subclones). Upon retesting all subclones again were classified the same as the parent strain.

Serum sensitivity and morphological variety. Figure 2 shows the distribution of serum sensitivity compared with morphological variety. The mucoid and dwarf varieties are more serum sensitive, whereas the gelatinous variety exhibits greater serum resistance. Sensitivities of multiple varieties from the same patient were then compared. Of 30 patients, 12 had a mucoid and a nonmucoid variety which differed by two or more sensitivity classifications (i.e., an S → IR, S → R, IS → R); in 6 patients, the two varieties were different Homma serotypes. All 12 patients had a more sensitive mucoid strain. Table 3 shows the distribution of sensitivity with respect to serotype. No apparent correlation could be demonstrated with the possible exception of type B. The eight strains of this serotype were all resistant or intermediate resistant; however, the small number of strains precludes the drawing of conclusions.

Nonmucoid revertant colonies were isolated from 15 mucoid strains and tested for serum sensitivity (Table 4). Revertant variants generally gave the same sensitivity as the parental mucoid (12 out of 15), whereas only 3 out of 15 were more resistant than the mucoid. Serotypes of the revertant morphology were the same as those of the mucoid morphology except for patients nos. 46 and 9.

Mechanism of bactericidal activity. CF strains of *P. aeruginosa* were tested in a variety of human sera to determine the requirement for killing these strains (Table 5). Bactericidal activity of both normal and CF sera was heat labile. No killing was observed in the presence of $MgCl_2$-EGTA-chelated serum, suggesting that only the classical pathway was able to initiate killing of these strains. Increased incubation periods up to 4 h in $MgCl_2$-EGTA-chelated serum

TABLE 1. *Comparison of serum sensitivity in normal and autologous CF sera*

Patient	Clinical condition[a]	Morphology[b]	Normal serum	Autologous CF serum
1	A	Muc	IS	IS
2	A	Rgh	S	S
		Cl	IS	IS
3	A	Rgh	R	R
4	A	Muc	S	S
5	A	Muc	R	R
6	A	Cl	S	S
7	A	Rgh	S	S
8	A	Muc	R	R
		Cl	R	R
		Rgh	S	S
9	A	Muc	S	S
10	A	Muc	R	R
		Rgh	IS	IS
11	A	Cl	S	S
12	A	Rgh	R	R
		Cl	IR	IS
13	A	Rgh	R	R
14	A	Muc	S	S
15	A	Muc	R	R
		Cl	R	R
16	A	Muc	IS	S
		Rgh	IR	S
17	A	Rgh	IS	IS
18	A	Muc	S	S
19	A	Muc	S	S
20	A	Cl	R	R
21	B	Muc	S	S
		Cl	IR	IS
22	B	Rgh	S	S
		Muc	S	S
23	B	Cl	R	R
24	B	Rgh	R	R
		Muc	S	S
25	B	Muc	IS	R
		Cl	S	IR
		Rgh	S	IR
26	B	Muc	R	R
		Rgh	R	R
27	B	Muc	R	S
		Cl	IS	IS
		Rgh	R	R
28	B	Rgh	R	IR
29	B	Muc	S	S
		Rgh	IR	IS
30	B	Rgh	S	S
31	C	Muc	S	R
		Cl	S	R
		Rgh	S	R
32	C	Muc	R	R
		Rgh	IS	IS
		Cl	IR	IR
		Gel	R	R
33	C	Rgh	IS	S
		Muc	S	S
34	C	Muc	IS	IS
		Rgh	IR	R
35	C	Muc	IS	S
		Cl	IS	S
36	C	Muc	S	S
		Rgh	IS	IR
37	C	Rgh	S	R
38	C	Muc	S	R
39	C	Cl	IR	IS
		Rgh	IR	IS
40	C	Muc	R	R
		Cl	R	R
		Rgh	S	S
41	C	Cl	IR	S
42	C	Rgh	S	S

[a] A, Good clinical condition; B, moderate clinical condition; C, poor clinical condition (see text for description). Patient age range was 9 to 36 years; median age was 18 years. All patients were colonized with *Pseudomonas* for at least 4 years.

[b] Muc, Mucoid; rgh, rough; cl, classical; gel, gelatinous.

did not change the sensitivity classifications, i.e., intermediate strains did not become sensitive. *E. coli* C was killed in all chelated sera, indicating that the alternate pathway was operative. The hemolytic complement levels in chelated sera were greater than 70 CH_{100} U/ml. A total of 23 sensitive CF strains were tested in chelated serum, and all were resistant. Absorption of CF serum with the autologous *P. aeruginosa* strain resulted in a decreased killing for four strains tested. Sera from two patients with blocking activity (nos. 31 and 38) were absorbed with the autologous strains, but no difference was observed in the bactericidal activity. Residual total complement levels as determined by immunodiffusion plates were unchanged in the absorbed sera, and the control *E. coli* C and a sensitive heterologous CF *P. aeruginosa* strain were both killed in the absorbed sera. Absorption of a control serum with strains from patients nos. 31 or 38 also did not alter its bactericidal activity against the autologous and heterologous *P. aeruginosa* or *E. coli* C. Two strains tested in hypogammaglobulinemic serum remained sensitive, whereas two others became more resistant.

DISCUSSION

The bactericidal activity present in human serum appears in vitro to be an effective mechanism for killing a significant number of *P. aeruginosa* strains from CF patients. A total of

46% of our CF isolates were determined to be sensitive, in contrast to *P. aeruginosa* from other clinical sources (21). It was expected that the serum resistance of an organism would contribute to its ability to colonize the CF lung. However, the mucoid and dwarf varieties, which are more often associated with severe clinical disease, are more serum sensitive than other varieties. A previous study on serum sensitivity reported no differences among mucoid and nonmucoid varieties (7); we can determine no apparent reason for this discrepancy other than possible differences in the patient populations, i.e., mode of therapy, duration of infection, or infecting organisms. Although the mucoid strains were often more serum sensitive, revertant colonies of these strains seldom changed sensitivity. Failure of the revertant strains to become more serum resistant suggests that the mucoid property is associated with sensitivity but does not determine serum sensitivity. Results of serum sensitivity testing of subclones indicate that little heterogeneity exists within a strain and that an intermediate strain is not a mixture of sensitive and resistant colonies.

TABLE 2. *Dilution of blocking serum into control bactericidal serum*

Dilution[a]	T_{60} colony counts[b] with serum from patient	
	No. 31	No. 38
Undiluted	320	296
1/2	ND[c]	>300
1/5	309	>300
1/10	178	>300
1/20	32	ND
1/50	4	20
1/100	0	17
Control serum	1	22

[a] CF sera from patients nos. 31 and 38 were diluted into the control serum. Undiluted control serum is shown on the last line. T_{60} colony counts without serum were >300.

[b] Of the autologous *P. aeruginosa* strain.

[c] ND, Not done.

The serum bactericidal activity against CF *P. aeruginosa* isolates appears to result from activation of the classical pathway since chelation of serum with $MgCl_2$-EGTA abolishes this activity. Increased time in chelated serum also did not alter the sensitivity of the intermediate strains, suggesting that these are not "delayed" sensitive strains (17), eventually capable of utilizing the alternate pathway. The *Pseudomonas* strains discussed here were capable of being killed only by the classical pathway, but a previous report (5) suggests that some strains of *Pseudomonas* may be capable of activating both complement pathways for opsonization. Conflicting reports of complement component levels in CF patients suggest that normal, elevated, or

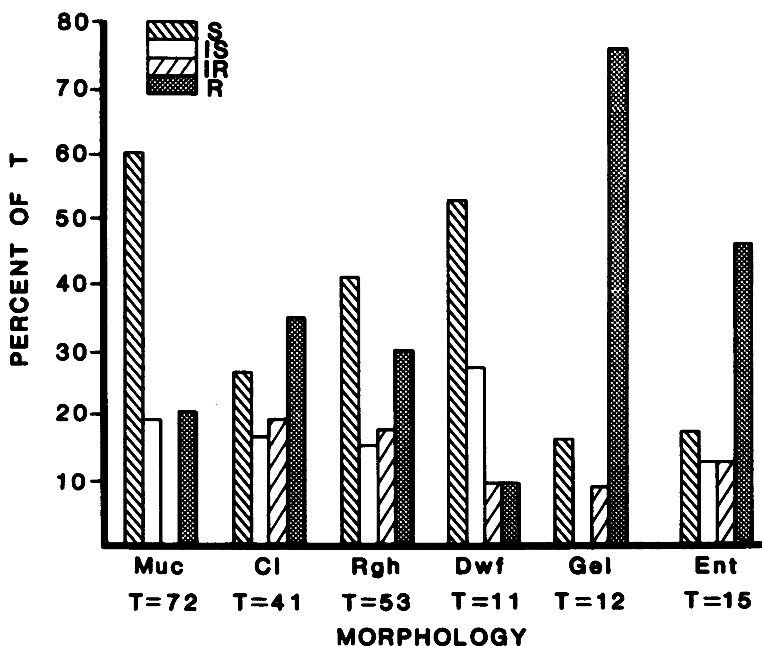

FIG. 2. *Distribution of serum sensitivity as a function of morphological variety of P. aeruginosa strains (T, total number of strains; muc, mucoid; cl, classic; rgh, rough; dwf, dwarf; gel, gelatinous; ent, enterobacter).*

decreased levels can be present (8), but these studies failed to consider the clinical status of the patient at the time of the study. All patients in our study had total complement levels within the normal range.

The decrease in killing with specifically absorbed serum and with hypogammaglobulemic serum suggests the requirement for antibody, although the amount of antibody needed was not determined. Normal serum contains sufficient levels of antibody to kill serum-sensitive *P. aeruginosa*. Presumably there is enough specific antibody in CF serum to support activation of the classical complement pathway. In some CF patients with advanced disease, however, a specific activity appears to protect the autologous strains from any serum bactericidal activity. The absorption procedure with the autologous strain failed to remove a detectable amount of the blocking activity from this serum, but possibly further absorption and dilution experiments may be necessary to elucidate this question. Addition of this serum to normal serum demonstrated that it could specifically block the bactericidal activity against the autologous strain. Similar effects have been reported in other systems (6, 19), and have been attributed to IgG. Fractionation of the serum from our patients with this blocking activity will be attempted when additional serum samples can be obtained.

At present, the serum sensitivity of a patient's colonizing strain cannot be correlated with any of the bacterial properties investigated here. However, the result of a strain's sensitivity in autologous serum compared to normal serum does correlate with clinical condition. Longitudinal studies including newly diagnosed patients are in progress to determine whether serum sensitivity is altered as the infection progresses. The appearance of strains resistant only in autologous serum from some patients in poor condition suggests that progression of the disease does influence serum sensitivity, but at present it is unclear when the transition may occur. The development of a blocking activity which renders the serum bactericidal activity inefficient for the autologous strain may signal a transition point in the progression of CF lung disease and may thus be another contributing factor in the failure of the CF host to control infection.

TABLE 3. *Serum sensitivity and Homma serotype of P. aeruginosa from 73 CF patients*

Homma type[a]	No. of strains (%)				
	S	IS	IR	R	Total
M	17 (49)	4 (11)	8 (23)	6 (17)	35
G	12 (55)	2 (9)	0 (0)	8 (36)	22
I	9 (36)	5 (20)	5 (20)	6 (24)	25
F	11 (61)	1 (6)	3 (17)	3 (17)	18
D	3	0	0	0	3
C	0	0	4	0	4
B	0	0	1	7	8
K	1	0	0	0	1
SA	3	0	0	0	3
NT	1	0	0	0	1
					120

[a] SA, Self-agglutinating; NT, nontypable.

TABLE 4. *Serum sensitivity with Difco and Homma serotype of mucoid and revertant strains*

Patient	Sensitivity of mucoid	Serotype[a]		Sensitivity of revertant	Serotype	
		Difco	Homma		Difco	Homma
43	S	SA	SA	S	SA	SA
8	R	10	H	IR	10	H
15	R	6	G	R	6	G
5	R	6	G	R	6	G
42	S	13	NT	R	13	NT
36	S	6	G	S	6	G
29	S	3	M	S	3	M
3	R	6	G	R	6	G
44	S	SA	SA	S	3	M
45	S	1	I	IR	1	I
46	S	6	G	R	1	I
47	R	6	G	R	6	G
48	S	14	M	S	14	M
49	S	9, 10	M	S	3, 9, 10	M
9	S	1	I	S	9, 10	NT

[a] SA, Self-agglutinating; NT, Nontypable.

TABLE 5. *Serum protein requirement for bactericidal activity against P. aeruginosa*

Patient	NS[a]	E-NS[b]	CFS	P.A. Abs[c] CFS	Hypo IgG[d]
11	S	R	S	IS	S
50	S	R	S	IS	S
4	S	R	S	R	IR
51	S	R	S	IS	R
31	S	ND[e]	R	R	ND
38	S	ND	R	R	ND
E. coli C	S	S	S	S	S

[a] NS, Normal serum.
[b] Normal serum chelated with $MgCl_2$-EGTA.
[c] CF serum absorbed with autologous *P. aeruginosa* strain.
[d] Hypogammaglobulinemic serum.
[e] ND, Not done.

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