Reprinted from THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, St. Louis
Vol. 97, No. 5, Pages 672-679, May, 1981 (Printed in the U. S. A.)

Detection of potential biochemical indicators of infection in the burned rat

MICHAEL C. POWANDA, JOHN DUBOIS, YSIDRO VILLARREAL, HARREL L. WALKER, and BASIL A. PRUITT, JR. *Fort Sam Houston, Texas*

Severe thermal injury is often complicated by infection. Moreover, the injury itself renders the early detection of infection more difficult. Rapid early detection of infection would thus aid in the treatment of severely burned patients. PCA filtrates of whole blood from burned-infected rats contain three substances that appear to be early indicators of infection in the thermally injured animal. These factors are only slightly affected by the extent of injury. These factors do not appear to be microorganism-specific in that they are found in rats infected with *Proteus mirabilis* as well as with *Pseudomonas aeruginosa*. One factor absorbs light at 398 nm and seems to be associated with some cellular component of blood. The other two substances are fluorescent, one λex 280 nm λem 340 nm, the other λex 355 nm λem 420 nm, and are detectable in PCA filtrates of plasma as well as of whole blood. All factors are retained by filters with a 25,000 dalton pore size. All factors are precipitable from PCA filtrates by phosphotungstic acid, suggesting that they may be proteins. The 355/420 factor increases with oxidation, whereas both the 280/340 substance and the 398 nm material decrease. (J LAB CLIN MED 97:672, 1981.)

Abbreviations: perchloric acid (PCA), optical density (OD), excitation wavelength (λex), emission wavelength (λem)

Severe extensive thermal injury is often complicated by the development of infection.[1] Severe thermal injury also complicates the detection of infection by altering the patient's febrile and leukocyte response,[2] and wound colonization can be mistaken for systemic infection[1] because wound manipulation can of itself induce transient bacteremia.[3] A simple, rapid early indicator of infection that requires small amounts of blood (<5 ml) and that does not respond appreciably to the extent of injury would significantly enhance care of the burn patient.

In the course of studies to determine whether a metabolic profile could be identified that would discriminate between burned and burned-infected rats,[4] we discovered that the native or background fluorescence of PCA filtrates of whole blood from burned-infected rats was greater than that from either control or burned-noninfected rats. Lloyd et al.,[5] who developed the analytic techniques we were using, had also noted that samples from very seriously ill patients possessed enhanced background fluorescence. We therefore pursued our observation and now describe the existence of three seemingly disparate substances that appear to be early indicators of infection in the injured host.

From the United States Army Institute of Surgical Research, Fort Sam Houston, Texas.
The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.
Submitted for publication Sept. 20, 1980; accepted Dec. 10, 1980.
Reprint requests: Library, U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas 78234.

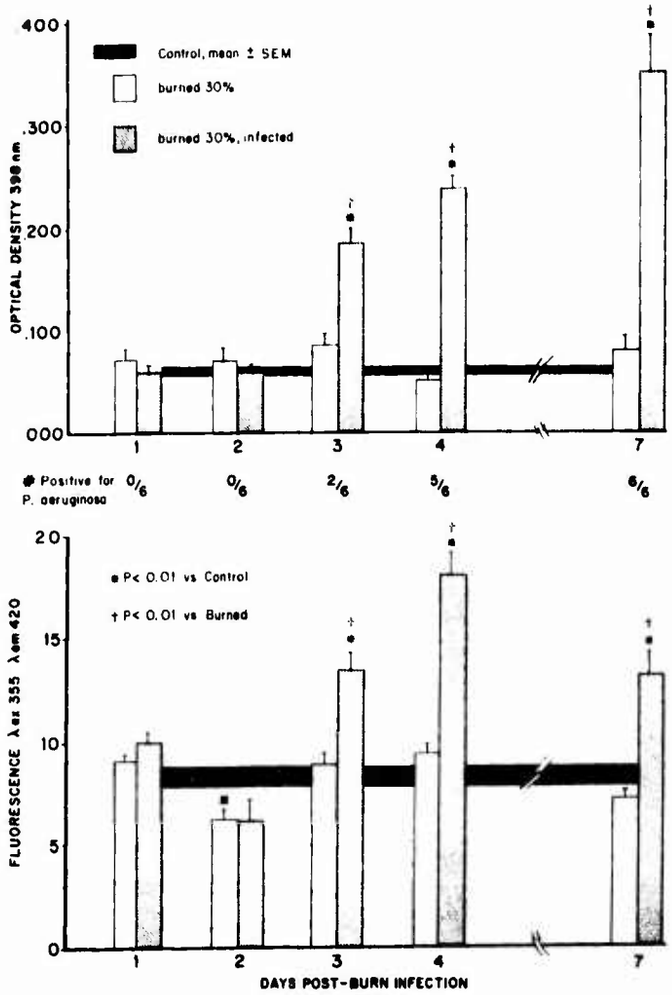

Fig. 1. Light absorption at 398 nm and fluorescence λex 350 nm λem 420 nm of PCA filtrates of whole blood. Mean ± S.E.M., n = 6.

Table 1. Light absorption (398 nm) and emission (420 nm) of PCA filtrates 4 days after burn/infection

	Absorption (OD)		*Emission (units)*	
	Whole blood	*Plasma*	*Whole blood*	*Plasma*
Control	0.063 ± 0.007	0.014 ± 0.004	2.4 ± 0.6	5.9 ± 0.9
Burned 30%	0.061 ± 0.007	0.008 ± 0.003	2.0 ± 0.4	5.1 ± 0.5
Burned 30%, infected	0.240 ± 0.032^{A,B}	0.010 ± 0.003	6.1 ± 0.8^{A,B}	19.8 ± 2.8^{A,B}
Burned 60%	0.072 ± 0.009	0.005 ± 0.002	4.3 ± 0.9	6.7 ± 0.9
Burned 60%, infected	0.356 ± 0.018^{A,C}	0.012 ± 0.004	12.3 ± 1.4^{A,C}	23.3 ± 2.7^{A,C}

Values are mean ± S.E.M.; n = 8. Infection was accomplished by swabbing 1 ml of a *P. aeruginosa* culture containing 10^8 bacteria on the dorsal surface within 1 hr of scalding.
p values: $^{A}p < 0.01$ vs. control; $^{B}p < 0.01$ vs. 30% burned; $^{C}p < 0.01$ vs 60% burned.

Table II. Effect of time, oxidation, and reduction on detection of biochemical indicators of infection

	Time of analysis (hr)			
	Untreated samples			*MSH-treated samples*
	2	4	24	2
OD 398 nm	0.509 ± 0.032	0.515 ± 0.023	0.428 ± 0.022	0.506 ± 0.030
355/420	22.6 ± 2.1	42.8 ± 2.4	88.2 ± 6.5	23.4 ± 2.2
280/340	2600 ± 225	1001 ± 91	818 ± 75	2500 ± 182

MSH (mercaptoethanol) and H_2O_2 were added after 2 hr reading.
Blood was taken from 30% burned-infected rats at 6 days, five rats were bled out into heparin (10 to 20 U/ml).

Methods

Male albino rats (180 to 200 gm) were used in all studies (Holtzman Co., Madison, Wisc.). In conducting the research described in this report, the investigators adhered to the *Guide for Laboratory Animal Facilities*, as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

A 30% total body surface full-thickness burn of the dorsum was achieved by immersing anesthetized (2.5 mg of sodium pentobarbital per 100 gm body weight), shaved rats, which had been placed in a mold to define the extent of injury, in boiling water for 10 sec.[6] No resuscitation was carried out. A 60% burn was achieved by immersing the ventral surface, as well, for 2 sec. Those rats with 60% burns were resuscitated with 20 ml of normal saline injected intraperitoneally. *Pseudomonas* infection was induced by placing 1 ml of a 16 hr broth culture on the dorsum of the 30% and 60% burned rats within 1 hr after scalding, followed by swabbing to distribute the organisms over the surface. A clinical isolate of *Pseudomonas aeruginosa* was used, strain 12-4-4, and the culture was adjusted to yield 10^8 organisms/ml. A clinical isolate of *Proteus*, strain 8-22-34, was also used, this time adjusted to 10^4 organisms/ml, and inoculation was delayed 6 hr after the burn so as to extend the time to death in this rapidly progressing disease. Blood samples from burned and burned-infected rats were cultured in trypticase soy broth to assess the presence of bacteria.

At the times required in each of the studies, the rats were anesthetized by the intraperitoneal injection of 0.5 to 1 mg of sodium pentobarbital per 100 gm body weight; the body cavities were opened, and blood was taken from the hepatic vein for detection of indicators and the presence of bacteria.

Detection of the putative biochemical indicators of infection was accomplished by mixing 1 ml of heparinized whole blood (10 to 20 U/ml heparin) with 4 ml of chilled 0.8M PCA in a 17 by 100 mm polypropylene tube. The mixture was allowed to stand for 10 min and then spun in a refrigerated centrifuge (Sorvall RC-3; Sorvall–DuPont Instruments, Wilmington, Del.) for 10 min at 2200 × g. The filtrates were poured into 12 by 75 mm polypropylene tubes and then spun at 48,000 × g for 20 min (Sorval RC 2-B), decanted into a second 12 by 75 mm tube and spun again at 48,000 × g for 20 min. Light absorption was measured with a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with 0.5M PCA as a blank, or a Beckman ratio recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.). The fluorometer was standardized by using a commercially available tetraphenylbutadiene standard. To assess statistical significance, the data were subjected to analysis of variance; in the case of data in Fig. 1, the least significant difference was used as the criterion, and in the case of the data in Table I, the Scheffé test.

Results

In the course of studies of metabolism in burned and burned-infected rats, it became apparent that PCA filtrates of whole blood from burned-infected rats had a greater

Time of analysis (hr)				
MSH-treated samples		*H_2O_2-treated samples*		
4	24	2	4	24
0.323 ± 0.034	0.403 ± 0.057	0.562 ± 0.028	0.090 ± 0.007	0.115 ± 0.001
82.3 ± 7.7	125.3 ± 10.8	23.3 ± 2.2	300.0 ± 21.4	479.2 ± 36.6
827 ± 57	605 ± 41	2292 ± 229	347 ± 45	237 ± 30

background fluorescence (λex 355 nm, λem 420 nm) than did those from burned or control rats. Filtrates from burned-infected rats, but not from burned-noninfected rats, also displayed a broad band of absorbing material with a peak from 394 to 402 nm when scanned in a dual-beam spectrophotometer; 398 nm was chosen to assay this factor.

Fig. 1 depicts a longitudinal study of the change in absorbance and fluorescence after injury with or without infection. Within 3 days of injury, there was a slight, but transient, increase in OD in samples from burned-noninfected rats. Samples from burned-infected rats displayed a significant increase in absorbance vs. both control and burned-noninfected rats on day 3 and additional increases in OD thereafter, so that by day 7 there was a sixfold difference in OD at 398 nm between samples from burned-infected and from either control or burned-noninfected rats. PCA filtrates from both burned-noninfected and burned-infected rats showed a modest decrease in fluorescence on day 2; samples from burned-noninfected rats displayed no subsequent change, but those from burned-infected animals contained significantly heightened fluorescence on days 3 through 7. These data underestimate the λex 355 λem 420, since H_2O_2 was not yet used to maximize detection of this factor. *Pseudomonas* bacteremia was detectable in 2/6, 5/6, and 6/6 burned-infected rats on days 3, 4, and 7, respectively. None of the 30% burned rats had positive blood cultures for *Pseudomonas*.

Table I indicates that the extent of injury produced no significant change in either absorbance at 398 nm or emission at 420 nm; however, infection overlaid on injury elicited a fourfold to sixfold increase in OD 398 and a threefold enhancement of fluorescence, again an underestimate. These data also indicate that the 398 nm absorbing material appears to be cell-associated whereas the fluorescence factor can be detected in PCA filtrates of either plasma or whole blood.

A closer examination of the fluorescence scans of PCA filtrates from control, burned-noninfected, and burned-infected rats indicates that in addition to the 355/420 factor, there appeared to be a 280/340 substance that increased with infection. Thus, in subsequent studies, this substance was also measured. It became apparent that emission at 420 nm increased but OD at 398 nm decreased if PCA-treated samples were allowed to stand for a few days at 4° C. This suggested that an oxidative process might have been occurring. Hydrogen peroxide was found to maximize the 355/420 fluorescence (Table II). The assay was then modified so that after readings at 398 nm and 280/340, 0.2 ml of 30% hydrogen peroxide was added and 1 hr later the 355/420 measurement was made. If whole blood samples were stored at 4° C and PCA added just prior to analyses, the indicators appeared to be stable for at least 3 days (Table III).

Preliminary evidence indicates that when PCA filtrates from burned-infected rats

Table III. Effect of storage of whole blood samples at 4° C

	Time of precipitation with 0.8M PCA (hr)				
	0	*4*	*24*	*48*	*72*
OD 398 nm:					
Control	0.065 ± 0.003	0.043 ± 0.003	0.030 ± 0.005	0.033 ± 0.005	0.034 ± 0.005
Burned	0.059 ± 0.006	0.030 ± 0.008	0.023 ± 0.005	0.018 ± 0.006	0.035 ± 0.003
Burned-infected	0.340 ± 0.042	0.376 ± 0.024	0.406 ± 0.025	0.326 ± 0.030	0.489 ± 0.031
280/340:					
Control	307 ± 5	303 ± 7	347 ± 25	250 ± 7	324 ± 46
Burned	478 ± 23	355 ± 9	363 ± 5	295 ± 25	438 ± 36
Burned-infected	1614 ± 93	1023 ± 53	1350 ± 49	1220 ± 57	1388 ± 48
355/420:					
Control	30.6 ± 0.8	26.8 ± 1.4	36.1 ± 1.8	35.3 ± 1.5	35.2 ± 3.7
Burned	34.8 ± 2.3	25.6 ± 2.1	35.3 ± 1.8	34.8 ± 2.0	40.8 ± 4.4
Burned-infected	214 ± 11	139 ± 5	173 ± 7	164 ± 8	198 ± 13

Eight rats per group were bled using heparin at 6 days after 30% burn ± infection (*P. aeruginosa*); 1 ml aliquots of blood were stored at 4° and precipitated with PCA at the times noted.

Table IV. Effect of filtration on detectability of indicators of infection

OD 398 nm		*λex 280 λem 340*		*λex 355 λem 420*	
Initial reading	*Filtrate reading*	*Initial reading*	*Filtrate reading*	*Initial reading*	*Filtrate reading*
0.359 ± 0.018	0.000	1700 ± 78	117 ± 3	147 ± 5	50 ± 3

Values are mean ± S.E.M.; n = 8.

Whole blood from 30% burned-infected rats (1 ml) was precipitated with PCA, and the absorbance and fluorescence of the supernatant were measured. The supernatant was then passed through an Amicon Centriflo filter (F 25) and the absorbance and fluorescence of the filtrate were measured.

were centrifuged through an Amicon Centriflo filter (Model F25; Amicon Corp., Lexington, Mass.) all the 398 nm material was retained but that 93% of the 280/340 and 66% of the 355/420 material was retained (Table IV). All factors were completely removed from PCA filtrates by the addition of 5% phosphotungstic acid in 2N HCl (Table V).

Alterations in these putative biochemical indicators of infection were not limited to *P. aeruginosa* infection. *Proteus mirabilis* infection in burned rats also induced changes in OD at 398 and in fluorescence (Table VI). By the second postburn, postseeding day, there was a doubling in the OD 398, and by 3 days a threefold to fourfold increase in OD was seen. As regards fluorescence, a twofold to threefold increase in 280/340 was seen at 2 days, and a similar increase noted at 3 days. In regard to the 355/420 factor, a twofold to threefold increase in it on day 2 and a threefold to fourfold increase on day 3 were found. Bacteremia was detectable in 2/6 infected rats on day 2 and in 3/3 on day 3. Thus, to some degree, the increase in biochemical indicators preceded sepsis in this model as well.

The results of analysis of samples of microorganisms for the presence of these indicators suggest that little or no 398 nm absorbing material or 280/340 fluorescent material was associated with either *P. aeruginosa* or *P. mirabilis* cultures (Table VII). *Pseudomonas* cultures did exhibit considerable 355/420 fluorescence, but it did not increase with the

Table V. Effect of phosphotungstic acid (PTA) treatment on detectability of indicators

OD 398 nm		*λex 280 λem 340*		*λex 355 λem 420*	
Initial reading	*After PTA*	*Initial reading*	*After PTA*	*Initial reading*	*After PTA*
0.361 ± 0.023	0.006 ± 0.002	1619 ± 77	0	146 ± 5	0

Values are mean ± S.E.M.; n = 8.
The absorbance and fluorescence of PCA filtrates was measured, and then 1 ml of PTA (5% in 2N HCl) was added; the filtrates were centrifuged, and absorbance and fluorescence were again measured.

Table VI. Alterations in biochemical indicators during infection with *Proteus* strain 8-22-34 in 30% burned rats

	Time (hr) after burn/infection		
	18 to 24 $n = 6$	*42 to 48* $n = 6$	*66 to 72* $n = 3$
OD 398 nm:			
Controls	0.061 ± 0.005	0.040 ± 0.007	0.040 ± 0.007
Burned	0.061 ± 0.006	0.064 ± 0.009	0.025 ± 0.003
Burned-infected	0.079 ± 0.001	0.116 ± 0.014	0.123 ± 0.026
280/340:			
Controls	218 ± 5	245 ± 10	277 ± 38
Burned	350 ± 23	510 ± 29	380 ± 6
Burned-infected	423 ± 22	1317 ± 86	1180 ± 180
355/420:			
Controls	19.2 ± 0.9	11.2 ± 1.2	19.7 ± 2.8
Burned	20.3 ± 1.7	22.7 ± 3.1	13.0 ± 2.5
Burned-infected	24.5 ± 1.5	62.0 ± 4.0	69.6 ± 22.7

addition of hydrogen peroxide, and most of it readily passed through an Amicon Centriflo filter. *Pseudomonas* cells contained no 398 nm material, a little 280/340 material compared to a saline blank, and negligible 355/420 fluorescence even after the addition of H_2O_2. *Pseudomonas* cells mixed with heparinized plasma contained minute amounts of absorbance at 398 nm, considerable 280/340 fluorescence, and some 355/420 fluorescence that increased somewhat upon the addition of H_2O_2, all apparently due to the presence of plasma. There appeared to be no additive or synergistic effects.

Discussion

The observation of increased native fluorescence in PCA filtrates of whole blood from burned-infected rats has led us to detect and describe three substances or sets of substances that can be rapidly analyzed (2 to 3 hr processing and analysis time) and appear to be sensitive, early indicators of infection in the burned rat. The origin of these indicators appears to be host-derived, for the most part. PCA filtrates of cultures of *Pseudomonas* and *Proteus* (approximately 1×10^9 microorganisms/ml) and washed *Pseudomonas* cells exhibit negligible absorption at 398 nm and fluorescence 280/340, suggesting that neither of these factors originates with the microorganism. Normal heparinized plasma has little or no absorbance at 398 nm, consistent with the apparent cell association of this factor. Heparinized plasma, however, does have normal levels of the 280/340 fluorescence. Ad-

Table VII. Analysis of microorganisms for the presence of indicators of infection

	OD 398 nm	*280/340*	*355/420*	*355/420 after H_2O_2*	*355/420 filtrate*[A]
P. aeruginosa 12-4-4:	0.003	0.5	94	70	54
Culture[B]	0.000	0	155	140	120
	0.000	0.5	160	120	105
Cells[C]	0.000	43	15	16	—
	0.000	43	16	15	—
	0.000	20	7	7.5	—
Cells + heparinized	0.008	950	15	33.5	—
serum[D]	0.004	930	15	32.0	—
	0.013	940	15	33.5	—
Heparinized serum	0.004	900	20	37	—
(0.5 ml) + saline (0.5 ml)	0.005	970	13	30	—
P. mirabilis 8-22-34 cul-	0.005	<0	<0	7	0
ture[B]	0.003	<0	<0	8	0
	0.004	<0	<0	7.5	0

[A] Amount of H_2O_2-treated material that passed through Centriflo filter.
[B] Three different 18 hr trypticase-soy broth cultures of each microorganism (approximately 1×10^9 microorganisms/ml) were tested; 1 ml of culture was mixed with 4 ml of 0.8M PCA. The original trypticase-soy culture medium was used as blank.
[C] Three different *P. aeruginosa* cultures were used; this time the cells were washed twice in saline and then resuspended in saline to yield 1 to 2×10^9 microorganisms/ml; 0.5 ml of resuspended cells was mixed with 0.5 ml of saline and then 4 ml 0.8M PCA. Saline was used as blank.
[D] A 0.5 ml volume of resuspended cells was mixed with 0.5 ml of heparinized plasma and then with 4 ml of 0.8M PCA. Saline was used as blank.

mixture of plasma and *Pseudomonas* cells yields no increase in λem 340 above that given by plasma alone. Normal plasma contains modest amounts of fluorescence 355/420 that doubles after the addition of H_2O_2. *Proteus* also contains minor amounts of fluorescence 355/420 detectable only in the presence of H_2O_2 but apparently retained by a Centriflo filter. *Pseudomonas* cultures, on the other hand, possessed considerable fluorescence 355/420, but unlike that found in PCA filtrates from infected animals, this did not increase with peroxide addition, and 70% to 85% of it passed through a Centriflo filter. This suggests that although *Pseudomonas* could give rise to some of the 355/420 material, there would have to be some change in its form such as would occur with aggregation or binding to a large molecule acting as a carrier, to account for the material being retained by molecular sieves when found in blood samples from burned-infected animals.

As to the nature of these factors, they are all soluble in 0.64M PCA (final concentration of mixture) and yet precipitated by the subsequent addition of phosphotungstic acid, which intimates that they have characteristics in common with the components of the seromucoid fraction. The seromucoid fraction, the concentration of which is a good indicator of the presence of inflammation and infection,[7, 8] contains α_1–acid glycoprotein, Gc-components, haptoglobin, hemopexin, β_2-glycoprotein, and a few minor components.[9] Both fluorescent factors are found in plasma and, as judged from filtration characteristics, have a minimum molecular weight of 10,000 in the case of the 355/420 substance and perhaps twice that for the 280/340 material. However, unlike the usual components of the seromucoid fraction, neither appears to change much with inflammation, at least in the case of burn injury. The 398 nm material is even more unlike a conventional carbohydrate-rich acute-phase protein in that it is found only in whole blood and not in plasma. Its molecular weight is at least 25,000 daltons. There is circumstantial

evidence that the 398 nm factor is proteinaceous, in that a positive linear correlation exists between the amount of protein retained by a Centriflo filter and the OD at 398 nm of the original sample. It seems clear that the 398 nm material is not equivalent to either the 280/340 or 355/420 substance. The 355/420 substance increases with oxidation, but the 280/340 material decreases; although this could be interpreted merely as a shift in molecular configuration resulting in new spectral characteristics, the 280/340 factor is 95% retained by a Centriflo filter, whereas a third of the 355/420 material passes through, which is circumstantial evidence of nonequivalence of these factors.

Whatever the ultimate identities of the factors, it seems clear that they respond primarily to the presence of infection and are not significantly affected by extent of injury. The analyses require 2 to 3 hr to perform, including processing time, and no special handling of the blood sample is required. The increases in these three indicators appear to parallel the development of systemic sepsis in these models. The initial increase in these three factors in many cases precedes the onset of bacteremia. Unlike the substances described by Baker et al.,[10] these factors confirm the presence of infection and monitor its progression rather than predict ensuing sepsis; they are thus akin to early acute-phase reactants.

Further studies of these putative indicators of infection in a compromised host will attempt to answer the following questions. (1) Can these factors be found in a burned host with a gram-positive infection? (2) What is the nature of these substances? Are they akin to acute-phase proteins? (3) Are these indicators of clinical value in the early detection of infection in burn patients? (4) Might these factors be of value in detecting infection in other instances of compromised host resistance, e.g., renal transplant patients, severely malnourished patients, or patients receiving chemotherapy or radiation therapy for cancer?

We appreciate the assistance of Douglas E. Mills in statistical analyses and Albert T. McManus in preparing cultures of microorganisms and determining bacteremias.

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