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IMMUNOMODULATION WITH KILLED *PROPIONIBACTERIUM ACNES* IN GUINEA PIGS SIMULTANEOUSLY VACCINATED WITH *BRUCELLA ABORTUS* STRAIN 19

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ABSTRACT

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Immunomodulation with killed *Propionibacterium acnes* was attempted in guinea pigs simultaneously vaccinated with *Brucella abortus* strain 19. Two groups, each comprised of 9 guinea pigs, were injected by different routes (s.c. and or i.v.) with 1.4 mg of *P. acnes* and 5 X 10^8 CFU of *B. abortus*, S-19, while 3 other groups each received either *P. acnes*, *B. abortus* S-19, or saline (s.c.). The antibody titers to *B. abortus* measured at 6, 10 and 14 weeks after vaccination indicated no significant ($P<0.01$) response in the 2 groups immunopotentiated with *P. acnes* concurrent with *B. abortus* S-19 vaccination. The delayed hypersensitivity response to 3 Brucella antigens conducted 8 weeks after immunization did not show a significant difference between the *B. abortus* S-19 vaccinated group compared with the 2 groups immunopotentiated and vaccinated. However, the proliferative response of lymphocytes to the *B. abortus* soluble antigen diluted 1:100 indicated significantly enhanced blastogenesis in the (s.c.) immunopotentiated and immunized guinea pigs compared with the *B. abortus* S-19 vaccinated group. A slightly enhanced response was also observed in the group immunopotentiated (i.v.) and vaccinated (s.c.). The guinea pigs were challenged with *B. abortus* strain 2308 and necropsied 4 weeks later. The mean splenic CFU of the Brucella in the group immunopotentiated (i.v.) and vaccinated (s.c.) was significantly decreased when compared with the guinea pigs vaccinated with *B. abortus* S-19 alone. These findings indicated that *P. acnes* administered simultaneously with *B. abortus* S-19 vaccine was able to augment the immune response in guinea pigs. Immunomodulation as evidenced by enhanced clearance of *B. abortus* from the spleens of immunopotentiated animals was presumably brought about by activated macrophages or a T-cell mediated cytolytic mechanism or both.

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INTRODUCTION

Immunomodulation with bacteria or their products have been effective in modulating certain cell-mediated immune responses such as tumor destruction (Castro, 1974a; Juy & Chedid, 1975) and activation of phagocytic cells for bactericidal activity (Briles et al., 1981). A striking feature of acquired cellular resistance to infection with certain facultative intracellular pathogens is their ability to elicit an immune response which provides protection against other unrelated bacteria (Ratzan et al., 1972). Well-documented studies include the infection of guinea pigs with Mycobacterium tuberculosis and their subsequent nonspecific resistance to infection with Brucella suis (Henderson, 1964). Similarly, mice infected with Brucella abortus were resistant to challenge with Listeria monocytogenes (Mackaness, 1964) and mice resistant to either Salmonella typhimurium or L. monocytogenes were resistant to challenge with either organism (Blanden et al., 1966).

Several studies (Filice et al., 1980; Kobayashi et al., 1980) have indicated that Propionibacterium acnes (earlier known as Corynebacterium parvum) is a potent stimulator of the humoral and cellular immune systems. In P. acnes-injected mice, splenomegaly and increased carbon clearance have been correlated with enhanced lymphoreticular proliferation (O'Neill et al., 1973). However, P. acnes has also been shown to augment the humoral immune response but to concomitantly depress certain T-cell mediated responses (O'Neill et al., 1973). Graft rejection (Castro, 1974b), reactivity to phytohemagglutinin (PHA), concanavalin -A, the mixed lymphocyte reaction and the graft versus host reaction all tend to be suppressed in P. acnes-treated mice (Scott, 1972). Results of several studies (Filice et al., 1980; Briles et al., 1981) suggest that P. acnes-induced augmentation of the immune response is associated primarily with macrophage activation. The characteristics of P. acnes activated macrophages include enhanced antigen processing (Wiener & Bandieri, 1975), increased phagocytic and bacteriostatic activity (Fauve & Herin, 1971) and accumulation of lysosomal enzymes (Olivotto & Bomford, 1974). Since macrophages play an important role in the immune response to facultative intracellular pathogens such as brucella, salmonella, listeria and mycobacteria, we believed that macrophages with an augmented microbicidal capacity would enhance the protection provided by B. abortus strain 19 (S-19) vaccination when simultaneously administered with P. acnes. The immunity conferred by B. abortus S-19 vaccine alone is relative (McDiarmid, 1957) and

varies with the challenge exposure. Therefore, we contend that B. abortus S-19 vaccine administered simultaneously with P. acnes bacterin would enhance the immune response to challenge-exposure with virulent B. abortus. The purpose of this study was to make a comparative evaluation of the immune response of guinea pigs to B. abortus when simultaneously administered with P. acnes. Guinea pigs were selected as a model in the present experiment since guinea pigs have served as the laboratory animal of choice, particularly for potency testing of vaccines against B. abortus (Thornton & Muskett, 1972), and for evaluating the immune response to experimental infection in several studies (Jones & Berman, 1971; 1975).

MATERIALS AND METHODS

Immunization of guinea pigs

Hartley strain guinea pigs (Ancare Corporation, Manhasset, NY) of both sexes weighing approximately 300 to 400 g were used. Group 1 guinea pigs (n=7) were injected subcutaneously (s.c.) with an 0.8 ml saline suspension containing 1.4 mg of killed P. acnes (C. parvum, Wellcome strain CN 6134, Wellcome Research Laboratories, Beckernham, England). Group 2 guinea pigs (n=7) were injected (s.c.) with 0.8 ml attenuated B. abortus S-19 vaccine containing approximately 5 X 10^8 colony-forming units (CFU). Group 3 guinea pigs (n=9) were injected (s.c.) adjacent to the axial lymph nodes a mixture comprised of both P. acnes 1.4 mg and B. abortus S-19 (5 X 10^8 CFU) in 0.8 ml saline. Group 4 guinea pigs (n=9) were injected with killed P. acnes (1.4 mg/0.4 ml) (i.v.) and B. abortus S-19 vaccine (5x10^8 CFU/0.8 ml) (s.c.). Group 5 guinea pigs (n=9) were given 0.8 ml of sterile saline (s.c.).

Humoral immune response

The humoral immune response was determined by the enzyme-linked immunosorbent assay (Ruppanner et al., 1980) with essential modifications. The titrations were conducted on microtiter plates (Cooke Microtiter System, Dynatech Laboratories, Inc., Alexandria, VA). B. abortus soluble antigen (BASA) (Berman et al., 1980) obtained from the National Veterinary Services Laboratory, Ames, IA, was diluted 1:200 with 0.06 M carbonate buffer (pH 9.6) and used to coat microtiter plates. Serum collected at 6, 10 and 14 weeks after immunization was titrated using 2 fold dilutions in duplicate. Antibody titers were also assessed on serum samples collected from each guinea pig 4 weeks following challenge with virulent B. abortus S-2308. Positive control

sera from a B. abortus hyperimmunized guinea pig and negative sera were included in each test. Horseradish peroxidase conjugated rabbit anti-guinea pig immunoglobulin G antiserum (Miles Laboratories Inc., Elkhart, IN) diluted 1:500 with phosphate buffered saline (pH 7.2) containing 0.5% Tween 20 was used as the second antibody and 2,2,-azino-di (3-ethylbenzthiazoline sulfonic acid),(Sigma Chemical Co., St. Louis, MO) was used as the indicator substrate in the ELISA test. The color reactions proportional to the bound antibody was graded visually on a scale of 1+ to 4+. The highest antibody dilution in which a 2+ reaction was present was recorded as the antibody titer of the test sample.

Delayed type hypersensitivity

The hair covering the lateral body wall was shaved with electric clippers, and 0.1 ml of each test antigen (BASA 1:100, Brucella cell surface protein (CSP) 1:100 and Brucella allergen (BA) 1:100) and saline was administered intradermally into separate sites. Injection sites were examined at 24, 48 and 72 hours for the presence of erythema and induration. Zones of induration exceeding 5 mm in diameter were recorded. Observations made at 48 hours were analysed statistically.

Lymphocyte blastogenesis

The proliferative response of lymphocytes in response to BASA and phytohemagglutinin (PHA) was assayed by the microculture technique described by Schultz and Adams (1978). Blood was collected from each guinea pig 4 weeks after challenge with virulent B. abortus. The mononuclear cells separated on a Ficoll-sodium diatrizoate (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) gradient were adjusted to an appropriate cell concentration and placed in flat-bottomed 96-well microculture plates (Costar, Broadway, Cambridge, MA). Tissue culture medium RPMI-1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 IU and streptomycin 10 mg/100 ml of medium) was used. B. abortus soluble antigen diluted 1:25, 1:100 and 1:400 in the same medium was used as specific antigen, while PHA at a dilution of 1:50 was used as mitogen. Negative control cultures without antigen or mitogen were set up in parallel. All cultures were incubated for 96 hours at 39^{o}C in a humidified atmosphere of air and 5% carbon dioxide. The cells were pulsed with 0.5 µCi of ^{3}H-thymidine (New England Nuclear Corp., Boston, MA), harvested by a MASH cell harvester onto

glass fiber filters (Microbiological Associates, Bethesda, MD), dried, and the counts per minute (cpm) of incorporated ^{3}H-thymidine was determined in a Beckman model LS-7000 scintillation spectrophotometer (Beckman Instruments, Inc., Irvine, CA). The counts per minute for the antigen and mitogen concentrations with each sample conducted in duplicate were recorded.

Challenge infection

Guinea pigs in each treatment group were challenged with virulent B. abortus S-2308 (obtained from the National Veterinary Services Laboratory, Ames, IA). Each guinea pig was injected intraperitoneally with 1 X 10^{6} CFU of B. abortus in 0.2 ml saline.

Enumeration of viable bacteria in the spleen

The number of viable bacteria in the splenic parenchyma was determined (Woodard et al., 1980) 4 weeks after challenge. The guinea pigs were necropsied, spleens were weighed and suspended (1.5 w/v) in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) and titurated in sterile Griffith's tubes. Appropriate decimal dilutions were plated in quadruplicate on trypticase soy agar plates and incubated at 37°C in a 10% CO_2 environment for 48 hours. B. abortus colonies were enumerated and representative isolates were biotyped. Previous studies with guinea pigs have demonstrated that S-19 does not survive in the tissues beyond 40 days (Mackaness, 1971).

Statistical analysis

Data from the experiment were analyzed by pairwise comparisons between the groups using the Student's t test.

RESULTS

Humoral antibody response

The serum antibody titers to B. abortus measured at 6, 10 and 14 weeks after vaccination and at termination of the experiment after the guinea pigs were challenged with the field strain 2308 are shown in Fig. 1. The antibody titers of the P. acnes injected guinea pigs (group 1) and the control guinea pigs (group 5) ranged between 1:8 and 1:32 during the first and second sampling periods. A slight elevation in titer was observed at 14 weeks post-treatment in both groups (Fig. 1), probably resulting from the skin test antigens administered to all groups at 8 weeks for measuring the DTH response.

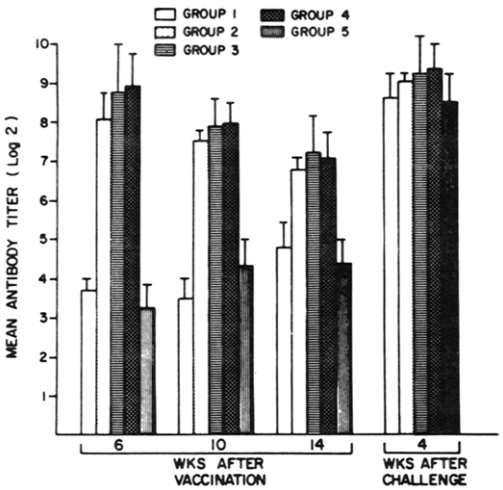

Fig. 1. Serum antibody titers for BASA measured by ELISA. Group 1, (6)* guinea pigs P. acnes, s.c. Group 2, (4) B. abortus, S-19, s.c. Group 3, (6) P. acnes, and B. abortus, S-19, s.c. Group 4, (9) P. acnes, i.v. B. abortus, S-19 s.c. Group 5, (5) Saline, s.c.

The antibody titers, however, were significantly (P>0.01) lower than those observed in the 3 vaccinated groups 2, 3 and 4. A slightly heightened antibody response was observed in the 2 groups (3 and 4) that were immunopotentiated with P. acnes concurrent with S-19 vaccination than in the group 2 guinea pigs that were immunized with S-19 alone. However, the increment in titers was not statistically (P>0.01) significant. A majority of the animals treated with P. acnes and S-19 had two-fold higher antibody titers than the group 2 guinea pigs that received S-19 only, thus indicating a slightly augmented antibody response.

Delayed type hypersensitivity response

The delayed dermal reactivity to 3 brucella antigen preparations was conducted at 8 weeks post-immunization. Zones of erythema at injected skin sites measured at 48 hours indicated no significant difference between the S-19 vaccinated group compared with the 2 groups immunopotentiated with P. acnes (Fig. 2). The frequency of positive (>5 mm) reactors to different brucella antigens was consistent. Some of the guinea pigs in group 1 and a few in group 5 that were not vaccinated with S-19 exhibited zones of erythema that were <5 mm; these reactions were considerably milder than those (>10 mm) in the groups 2, 3 and 4 that were vaccinated with S-19 prior to skin testing.

All antigens effectively distinguished responses in vaccinated versus nonvaccinated guinea pigs.

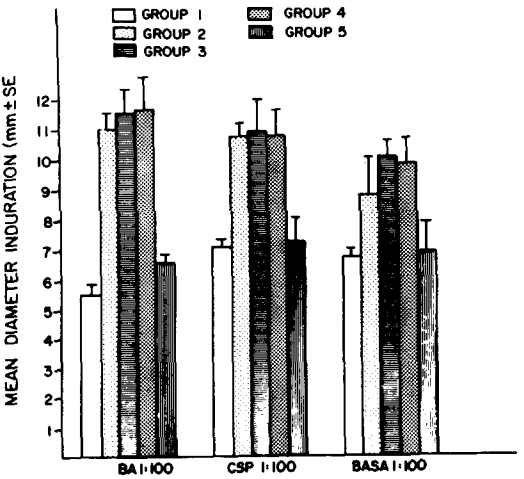

Fig. 2. Delayed hypersensitivity skin reactions to Brucella allergen (BA), Brucella cell surface protein (CSP), and B. abortus soluble antigen (BASA) measured 24 h. after injection. Group 1, (6)* guinea pigs P. acnes, s.c. Group 2 (4) B. abortus, S-19, s.c. Group 3 (6) P. acnes and B. abortus, S-19, s.c. and Group 4 (9) P. acnes i.v., B. abortus, S-19, s.c. and Group 5 (5) Saline, s.c.

Lymphocyte blast transformation response

The proliferative response of lymphocytes to 3 concentrations of BASA and PHA of all groups is shown in Table 1. Due to fatalities that occurred from bleeding attempts during the course of the experiment, the blast transformation test was conducted on cardiac blood drawn from each guinea pig at termination of the experiment. The results indicated a two-fold greater response in the lymphocyte cultures from group 3 when compared with group 2 guinea pigs at a BASA dilution of 1:100 (Table 1). A slightly enhanced response was also observed in group 4 guinea pigs; however, this increase was not significant ($P>0.01$). The lymphoproliferative response of groups 3 and 4 were significantly ($P<0.01$) greater than the nonvaccinated group 1 that was injected P. acnes only (Table 1.) The lymphoproliferative response to PHA did not show any significant difference ($P<0.01$) between the groups. The significantly higher lymphocyte blast response to BASA in group 3 guinea pigs and a moderately augmented response in group 4 guinea pigs reflect an

TABLE 1

Proliferative response (cpm) of lymphocytes to B. abortus soluble antigen (BASA) and phytohemagglutinin (PHA)

(No. of animals)	Treatment	Stimulation by BASA 1:25	BASA 1:100	BASA 1:400	PHA 1:50	Control Unstimulated
1. (6)	P. acnes s.c.	1,278 ± 334 (53 ± 475)*	1,828 ± 441 (603 ± 595)[a,b]	1,670 ± 399 (445 ± 432)[e,f]**	29,825 ± 9,107 (28,600 ± 8,736)	1,225 ± 428
2. (4)	B. abortus s.c.	3,038 ± 459 (1,908 ± 491)	4,238 ± 186 (3,108 ± 455)[c]	3,155 ± 242 (2,025 ± 241)	34,413 ± 4,405 (33,283 ± 4,925)	1,130 ± 384
3. (6)	P. acnes s.c. B. abortus S-19 s.c.	3,090 ± 729 (1,923 ± 589)	7,785 ± 844 (6,618 ± 671)[b,c,d]	5,060 ± 599 (3,893 ± 744)[e]	54,783 ± 5,866 (53,616 ± 5,782)	1,167 ± 192
4. (6)	P. acnes i.v. B. abortus S-19 s.c.	2,895 ± 1,135 (1,465 ± 933)	6,863 ± 918 (5,433 ± 737)[a]	3,917 ± 548 (2,487 ± 408)[f]	42,313 ± 4,110 (40,833 ± 3,965)	1,430 ± 291
5. (4)	Saline s.c. (control)	1,550 ± 283 (90 ± 149)	3,318 ± 994 (1,858 ± 981)[d]	3,768 ± 876 (2,308 ± 779)	37,180 ± 2,332 (35,720 ± 6,831)	1,460 ± 247

* No. in parenthesis represents Δcpm (cpm per 10^5 cells in BASA or PHA stimulated cultures minus cpm per 10^5 cells in unstimulated cultures) ± standard error.

** Values in the same column with the same letter are significantly ($P < 0.01$) different from one another.

enhancement of the lymphocyte response in P. acnes S-19-treated guinea pigs than in animals treated with S-19 alone.

Viable bacteria in the spleen

The number of viable B. abortus isolated from the splenic parenchyma of the surviving guinea pigs in the 5 treatment groups are presented in Table 2. The highest mean CFU were observed in the unvaccinated groups 1 and 5 while the lowest mean CFU were recorded in the immunopotentiated groups 3 and 4. A significant decrease ($P<0.01$) in the mean splenic CFU was observed in the group 4 guinea pigs, compared to the group 2 guinea pigs that received S-19 only. Group 3 guinea pigs that were immunopotentiated with P. acnes by the (s.c.) route did not show a significant reduction in the number of brucellae isolated from the spleen.

TABLE 2.

Colony forming units (cfu) of viable B. abortus S-2308 in splenic tissue

	No. of animals	Avg. Brucellae/ spleen x 10^7	Avg. spleen wt. gm.
1. P. acnes s.c.	5	1.33± 0.677*	4.08
2. B. abortus S-19 s.c.	4	0.21 ± 0.04	5.01
3. P. acnes s.c. B. abortus S-19 s.c.	6	0.13 ± 0.14	9.32
4. P. acnes i.v. B. abortus S-19 s.c.	8	0.08 ± 0.19**	10.10
5. Saline (control) s.c.	4	1.4 ± 0.432	4.91

*Standard error of mean
**Significantly different ($P<0.01$) compared to group 2

DISCUSSION

Modulation of the immune response to B. abortus S-19 vaccine in guinea pigs simultaneously injected with P. acnes indicated an augmentation of some of the immune responses examined. A slight increase in the serum antibody titers was observed in group 4 guinea pigs when compared with group 2 which received S-19

only. Although an increase in antibody titers were also observed in group 3 guinea pigs (Fig. 1) which were immunized (s.c.) with P. acnes and S-19, the mean antibody titers were not significantly different when compared with the titers of guinea pigs injected with S-19 alone. Thus indicating that the humoral antibody response was not appreciably altered when S-19 was administered with P. acnes. In accord with studies (Mackaness, 1971; McGregor & Kostiala, 1976) on many facultative intracellular bacteria, the antibody response to B. abortus is generally not considered to correlate with protective immunity (Sutherland, 1980). Nevertheless, opsonization has generally been accepted as a prerequisite for phagocytosis by macrophages and neutrophils. In the present study, clearcut evidence for such a mechanism is lacking, although an augmented clearance of B. abortus from the spleens of group 4 guinea pigs was observed (Table 2). In rabbits immunized with B. melitensis, Rev 1 vaccine, it has been observed that both serum and macrophages play a role in optimal inhibition of virulent B. melitensis (Ralston & Elberg, 1969;1971). A positive correlation between antibodies to brucella and protective immunity against challenge infection has also been demonstrated in mice (Bascoul et al., 1978; Plommet & Plommet, 1983).

With many facultative intracellular bacteria such as brucellae, a predominant cell-mediated immune response where immune T-cells play a significant role has been demonstrated (Jones & Buening, 1983). In the present study, the cutaneous DTH response to 3 different brucella antigens was not significantly ($P<0.01$) different in the 2 immunopotentiated groups 3 and 4, compared with the response of guinea pigs treated with S-19 alone (Fig. 2). The zones of induration observed with all 3 antigen preparations were comparable and consistent with skin test responses observed by other workers (Jones & Berman, 1971; Thornton & Muskett, 1972) on B. abortus infected or vaccinated guinea pigs. Contrary to the present findings, Collins and Scott (1974) observed a marked impairment of the DTH response to Salmonella enteritidis in mice pretreated with 700 µg of killed P. acnes and subsequently challenged with S. enteritidis 7 days later. However, Collins and Scott (1974) attributed the enhanced clearance of S. enteritidis in P. acnes pre-treated mice to phagocytosis by activated macrophages. Other studies (Bomford, 1980) have demonstrated a consistent DTH response to sheep RBC in mice and to ovalbumin in guinea pigs when P. acnes was previously injected via the foot pad. Recently, Briles et al., (1981) showed that augmented clearance of S. typhimurium by peritoneal macrophages of P. acnes treated mice was

associated with a T-cell mediated phenomenon because athymic nu/nu mice were refractory to similar treatment.

In contrast to the similarity in the cutaneous DTH response among the vaccinated and immunopotentiated/S-19 treated groups, the in vitro lymphocyte blast transformation response indicated a significantly enhanced stimulation in group 3 compared to group 2 guinea pigs (Table 1). A moderately enhanced response was also observed in group 4 guinea pigs. Experiments on mice injected with P. acnes have indicated an impairment of the in vitro response of lymphocytes to PHA (Scott, 1972). However, parenteral injection of P. acnes into P. acnes monoassociated rats has resulted in an increased splenocyte blastogenic response to PHA and Con A but not to homologous (P. acnes) antigen (Wells & Balish, 1979). In vivo studies with guinea pigs have also demonstrated a great expansion of the lymphocyte population within the para-cortex (thymus-dependent area) followed by extensive accumulations of lymphocytes in the sinuses. Although evidence in the present study is meager, it is plausible that P. acnes augmented the CMI response in S-19 vaccinated guinea pigs.

The comparative assessment of the number of residual brucellae in the spleen is perhaps the most unequivocal criterion for evaluating protection between the different treatment regimens. A significant reduction in the number of B. abortus in the splenic parenchyma of group 4 guinea pigs injected (i.v.) with P. acnes followed by (s.c.) injection of S-19 demonstrated an augmented clearance of the challenge organism. Overwhelming evidence from similar studies (Filice et al., 1980; Briles et al., 1981) lends to the conclusion that activated macrophages in P. acnes treated animals play an important role in the phagocytosis and clearance of organisms.

Observations made by investigators who have studied the cellular components of P. acnes involved in its immunomodulating activity have led to different conclusions (Riveros-Moreno & Niblock, 1979; Pringle & Cummins, 1982). Schwab (1975) has suggested that the effects of P. acnes as an adjuvant for humoral and cellular immune responses are triggered by more than one component of the bacterial cell. In addition to macrophage phagocytic activity, we postulate that other CMI responses such as antibody dependent cellular cytotoxicity and direct cell mediated cytolysis may occur consequent to P. acnes induced immunomodulation. The observation (Fisher et al., 1974) that P. acnes administered to tumor bearing mice were able to augment the in vitro cytotoxicity of lymph node cells and recent evidence (Nencioni et al., 1983)

that lymphoid cells are involved in the destruction of S. typhimurium by natural and antibody dependent cell mediated cytotoxicity support such a mechanism.

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